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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

III. A Second Type of Salmonella Naturally Appearing in the Endemic Stage.

By JOHN B. NELSON, Ph.D.

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(Received for publication, June 18, 1927.)

The course of a natural outbreak of paratyphoid disease in a guinea pig population was outlined in a preceding paper.[1] The active stage, which occupied a period of 8 weeks in the summer of 1924, was followed by a long endemic stage characterized by sporadic deaths. A single type of *Bacillus paratyphi* was held to be the agent throughout both stages. Continued investigation showed that a second, serologically different type had come into the population during the summer of 1926. The disease was still endemic at the time. The uniformity of the guinea pig stock had not been altered, meanwhile, by the addition of animals from outside sources. Mention should be made here of a similar occurrence reported by Lynch[2] who described the spontaneous appearance of a second Salmonella type in a mouse population. Further reference to the report will be given in a subsequent paper.

Since the introduction of specific infection into the guinea pig population all animals that died from natural causes have been autopsied. In all cases a bacteriological examination, generally of the spleen alone, has been made. Previous experience had shown that the spleen yielded a positive culture, in the majority of cases, whether the animal was a carrier or in a state of active disease. Cultures that were presumptively identified as belonging to the Salmonella group were tested by direct agglutination with a specific antiserum. From

[1] Nelson, J. B., and Smith, T., *J. Exp. Med.*, 1927, xlv, 353.

[2] Lynch, C. J., *J. Exp. Med.*, 1922, xxxvi, 15.

the onset of the epidemic up to the summer of 1926, the strains isolated from active cases and from carriers had shown sufficiently uniform agglutination reactions to be regarded as individuals of one general type. One serum employed in identification agglutinated its homologous strain through a dilution of 1:51,200. With this serum the strains examined agglutinated either to the titer limit or in the next lowest dilution, 1:25,600. It is realized that conclusions based on the outcome of direct agglutination tests alone may be misleading. The serum in question, however, was tested against a comprehensive series of other members of the Salmonella group and found to be low in group agglutinins. Of the types examined none agglutinated in a dilution higher than 1:800. Even though the isolated strains were not subjected to a more exact analysis by absorption it is believed that the position taken as to their unity is secure.

The first culture of the second type was isolated from a breeding sow, on July 28, 1926. At autopsy the animal showed a congested spleen. Focal lesions were not visible grossly. Peyer's patches of the small intestine were prominent and likewise congested. From the spleen there was obtained a motile bacillus which produced hydrogen sulfide and which failed to ferment either lactose or saccharose. Tested with the stock antiserum it agglutinated only in low dilution, 1:400. The following month three additional spleen cultures were isolated. These gave identical findings. Two of the guinea pigs were unweaned young. Only one showed suggestive lesions, the presence of small white plaques in the cecum and a congested spleen. The third culture was from a young, weaned guinea pig which showed an enlarged spleen together with minute focal lesions.

Animal injection was resorted to as a preliminary measure in the identification of these strains.

1 cc. amounts representing a 1:4,000 dilution of an 18 hour bouillon culture were injected intraperitoneally into 350 gm. guinea pigs. A single animal was employed for each strain. After 14 days the four guinea pigs were chloroformed and autopsied. In each case the postmortem findings were typical of paratyphoid infection. There was, however, a considerable individual variation in the type and extent of the changes produced. All showed enlarged spleens partially covered by an exudative membrane. There were no foci. Two showed purulent fluid in the gall bladder accompanied in one case by scattered liver foci. The

third showed focal lesions alone, and with the fourth the liver was normal. Three showed focal lesions in the lymphoid tissue of the small intestine, while the fourth showed small white plaques in the cecum. Pure cultures of an organism possessing characters identical with those of the injected culture were obtained from the spleen in each case. It seemed evident from the above findings that the four cultures were Salmonella types.

Rabbits were given serial intraperitoneal injections of heated killed suspensions of two strains for the production of antiserums. The two immune serums agglutinated the four cultures equally through a dilution of 1:12,800. A strain of the first type was agglutinated in low dilution only, 1:800. The absorptive capacities of the two immunizing strains as tested by reciprocal agglutinin absorption were identical. The above results in conjunction with the animal tests establish the position of the four cultures as identical strains of a second Salmonella type. From August on sporadic deaths due to one or the other of the two types, provisionally designated *B. paratyphi* Types I and II, have occurred. The relative distribution of the two types within the population will be considered in another paper. It may be said that 87 cases of the second type have been observed and studied during a period of 10 months.

In addition to the different agglutinative relationships of the two types of *B. paratyphi*, there was some indication that their loci of development in the animal host tended to differ. As previously noted,[1] the most constant manifestation encountered in fatal cases of the earlier type was the presence of focal lesions in the spleen together with enlargement and congestion. Focal involvement of the lymphoid tissue of the small intestine was often observed and somewhat less frequently of the liver. With the introduction of the second type of infection the number of cases showing typical focal lesions in the spleen has decreased. Thus, from the autopsy records of 50 consecutive cases of each of the two types, focal lesions were observed 29 times with Type I and 17 times with Type II. When present in the latter type, however, the focal changes showed no difference grossly from those of the earlier form of infection. In the absence of foci, congestion and slight enlargement of the spleen were commonly observed. Focal involvement of the Peyer's patches of the small intestine was also less frequent. In the liver, on the other hand, it

was more often observed than in Type I cases. Lesions in the cecum have likewise been more frequent with the Type II infection. The wall of the cecum was generally congested. At times the congestion was diffuse, involving the entire wall or extensive areas of it. At times it was circumscribed, involving only the lymphoid tissue which stood out as deep red circular patches. In addition, yellowish or yellowish white plaques varying in number and in size were present. Sometimes the plaques were located on the peritoneal surface, sometimes on the mucous surface. At times a diffuse exudate coated the mucous surface giving a dense opaque appearance. Frequently the cecal contents was fluid or semifluid.

Other gross changes noted at times in individual cases included involvement of the cervical and mesenteric lymph nodes with hypertrophy and congestion, a purulent fluid in the gall bladder, and firm adhesions binding the liver or spleen to the peritoneal wall. In several instances an acute peritonitis was encountered. These cases, which were limited to the stock, weaned guinea pigs, were marked by a tenacious exudative membrane on the surface of the liver and spleen, a seromucoid exudate coating the intestinal tract and peritoneum, and a considerable volume of turbid, mucoid fluid in the abdominal cavity.

Involvement of the genital tract was often observed in the case of adult females from the breeding cages. The uterine wall was swollen and diffusely congested. At times a thick, mucopurulent exudate was present in the lumen. At times small exudative plaques were found on the peritoneal surface of the uterus. In several instances an abscessed condition of the mammary gland was encountered. In young female guinea pigs uterine changes aside from a slight congestion were not conspicuous. Involvement of the male genital tract was rarely encountered.

The relationship of the second type of *B. paratyphi* to other members of the Salmonella group was studied by means of direct agglutination and by agglutinin absorption. An antiserum against Strain 1149, the first Type II culture isolated, was employed in the direct agglutination tests. The antigens were fresh, unheated, saline suspensions prepared from 18 hour agar cultures and were standardized to equal opacity. The agglutination limits of Salmonella types and

one strain of *B. typhi* with the Type II antiserum are given in Table I. Of the cultures employed, guinea pig Strains 1149 and 922 were Types II and I, respectively, from the present epidemic. Guinea pig Strain IV was isolated during a Boston epidemic in 1908. As noted in a previous paper,[1] it was agglutinated by a Type I serum in low dilution only. The rabbit strains were isolated during a slight outbreak among the stock rabbits in the fall of 1926. The mouse cultures were Mouse Typhoid I and II from The Rockefeller Institute in

TABLE I.

Agglutination Limits with Type I and Type II Antiserums.

	Antiserum	
	Type I	Type II
Guinea pig paratyphoid 922	1:51,200	1:800
" " " 1149	1:400	1:12,800
" " " IV	1:200	1:12,800
Rabbit " 5	1:51,200	1:400
" " 22	1:400	1:12,800
Mouse " I	1:51,200	1:800
" " II	1:200	1:12,800
Rat " V	1:51,200	1:800
Calf " I	1:400	1:12,800
Swine " IV	1:200	1:12,800
B. paratyphi a Schottmüller	1:200	1:800
" " b Rowland	1:200	1:6,400
" " *aertrycke* 387	1:200	1:12,800
" *enteritidis* Gaertner (Kral)	1:400	1:400
" *choleræ suis* X	1:100	No agglutination at 1:100
" *typhi* X	1:3,200	1:3,200

New York. The Rowland strain of *B. paratyphi* b was obtained through the courtesy of Miss Georgia Cooper of the New York City Department of Health. The other cultures were selected from the departmental collection.

The Type II serum agglutinated a number of the miscellaneous Salmonella cultures to the titer limit. Both of the serums were high in group agglutinins for *B. typhi*, while both were low for *B. enteritidis* and *B. choleræ suis*. The level of group agglutination for the opposite

type was somewhat higher in the case of the Type II serum. In the routine examination of cultures from positive cases of paratyphoid the limit of group agglutination of the Type II serum with the opposite type, however, varied from 1:200 to 1:800. The Salmonella cultures which showed a high agglutination with the Type II serum were selected for further identification by agglutinin absorption In each case the serum in a dilution of 1:25 was absorbed twice with a 1:10 volume of packed and washed cells. The absorption was carried out at 37°C. for 5 hours, followed by approximately 16 hours at ice box temperature. The absorbed serum was finally tested in a two-fold dilution series ranging from 1:50 through 1:25,600. The results

TABLE II.

Agglutination Limits with Type II Serum after Absorption.

Absorbing culture	Agglutination after absorption	
	Absorbing culture	Homologous culture
Guinea pig paratyphoid 1149		1:100
" " " IV	1:200	1:200
Rabbit " 22	1:100	1:100
Mouse " II	1:50	1:50
Calf " I	1:200	1:200
Swine " IV	1:100	1:1,600
B. paratyphi aertrycke	1:100	1:200
" " b Rowland	1:50	1:6,400

of the absorption tests with the Type II serum are given in Table II. Absorption with the homologous strain lowered the agglutinin content of the serum to the same level for all the cultures.

The absorptive capacity of the guinea pig culture, Strain IV; the mouse culture, Type II; the calf culture, Strain I; and the *aertrycke* type of *B. paratyphi* corresponded very closely to that of the homologous culture. By the direct method these cultures were agglutinated in low dilution only by the Type I serum. A close relationship between them and the Type II organism is indicated. The absorptive capacity of *B. paratyphi* b was approximately half that of the homologous culture. The reduction in titer of the serum for the

Type II antigen after absorption with *B. paratyphi* b was probably due to the removal of group and not specific agglutinin. By the direct method the culture was agglutinated in high dilution. The difference in absorptive capacity of the two cultures appears sufficiently marked to establish their non-identity. The Swine IV antigen reduced the titer of the serum approximately 87 per cent as compared

TABLE III.

Agglutination Limits with Mouse II, Calf I, and B. paratyphi aertrycke Serums after Absorption.

Serum	Culture	Direct agglutination	Absorbing culture	Agglutination after absorption	Culture agglutinated
Mouse II	Mouse II	1:51,200	Mouse II	1:100	Mouse II
			" II	1:100	Guinea Pig 1149
	Guinea Pig 1149	1:51,200	Guinea Pig 1149	1:100	Mouse II
			Guinea Pig 1149	1:100	Guinea Pig 1149
Calf I	Calf I	1:51,200	Calf I	<1:50	Calf I
			" I	<1:50	Guinea Pig 1149
	Guinea Pig 1149	1:25,600	Guinea Pig 1149	<1:50	Calf I
			Guinea Pig 1149	<1:50	Guinea Pig 1149
B. paratyphi aertrycke	*B. paratyphi aertrycke*	1:25,600	*B. paratyphi aertrycke*	1:50	*B. paratyphi aertrycke*
			B. paratyphi aertrycke	1:50	Guinea Pig 1149
	Guinea Pig 1149	1:25,600	Guinea Pig 1149	1:50	*B. paratyphi aertrycke*
			Guinea Pig 1149	1:50	Guinea Pig 1149

with the 99 per cent reduction by the homologous antigen. It seems necessary to assume that some specific agglutinin was removed and that the two cultures bear a relationship other than through their group agglutinogen.

In order to establish the identity or close relationship of the cultures which showed nearly equal absorptive capacities reciprocal

tests were made. The Calf I, Mouse II, and *aertrycke* cultures were employed. Rabbits were immunized with the three antigens and agglutinating serums of high titer obtained. The period of immunization was longer than employed in the preparation of the Type II serum and the titer limits of the serums were correspondingly higher. Absorption was carried out, as before, with the homologous culture and with the Type II culture. The results of the absorption tests are given in Table III.

The agglutinative affinities of the second organism as established by reciprocal absorption relate it to the *aertrycke* type of *B. paratyphi*. The identity of the Type II culture, the Mouse II culture, and the Calf I culture is similarly indicated. The identity of the Type II culture, the Guinea Pig IV culture, and the Rabbit 22 culture, while unconfirmed, is suggested. The Swine IV culture appears to be a less closely related organism, but bearing some specific agglutinogen in common with it. The second organism is related to the Type I strains, *B. enteritidis*, *B. paratyphi* a and b only through group agglutinogen. The content of group agglutinogen is low except for the latter organism from which it is differentiated only by agglutinin absorption. It also bears group agglutinogen in common with *B. typhi*. The indicated relationship of the Guinea Pig II, Mouse II, and *B. paratyphi aertrycke* strains is in agreement with the work of Webster[3] and of Edwards and Rettger.[4]

SUMMARY.

The spontaneous appearance of a second paratyphoid infection in a guinea pig population during the endemic stage of an earlier epidemic is reported. A comparative study of the gross pathology of the two infections indicated a difference in the loci of development of the respective organisms in the animal host. The two types were readily differentiated by direct agglutination with specific immune serums. From its agglutinative affinities the second organism was judged to be an *aertrycke* type of *B. paratyphi*.

[3] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

[4] Edwards, P. R., and Rettger, L. F., *J. Bact.*, 1927, xiii, 73.

VARIATIONS IN THE SCOURS TYPE OF BACILLUS COLI FROM THE STANDPOINT OF BACTERIOPHAGIC ACTION.

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Certain strains of the scours type of *Bacillus coli*, described by T. Smith and Bryant,[1] are subject to variation, on solid media, characterized by the appearance of indented areas at the periphery of the colonies. These areas may be v-shaped or hemispherical and are underlaid by a secondary growth which differs from the primary, mucoid, opaque form in that it is non-mucoid and translucent. The bacteria comprising it are actively motile whereas those of the parent colony are non-motile or sluggishly so. Both forms produce a diffuse turbidity in bouillon. Subcultures made from the secondary growth are pure with respect to the characters enumerated and continue to breed true in subsequent generations. Subcultures from the parent colony are unstable and subject to variation under certain conditions.

Hadley[2] describes a somewhat similar type of variation occurring in colonies of Friedländer's pneumobacillus and of various intestinal bacteria marked by the appearance of peripheral, bluish, translucent invaginations. These may extend radially to produce a fringe or backward into the colony which may eventually be wholly consumed. The secondary growth is comprised largely of the R type of culture and upon subculture yields thin, irregular, translucent colonies. This form of variation, termed by Hadley marginal dissociation, may occur suddenly and spontaneously in cultures which have previously given a normal growth on solid media. Its analogy with the present form of variation appears close but not complete. With the colonies of the

[1] Smith, T., and Bryant, G., *J. Exp. Med.*, 1927, xlvi, 133.

[2] Hadley, P., *J. Infect. Dis.*, 1927, xl, 1.

scours type of *B. coli* the peripheral indentations are always present in the initial culture, made directly from the calf. They are to be observed in the majority of the colonies. The exceptions, which appear normal in that generation, invariably undergo variation upon subculture. The secondary growth, unlike that described by Hadley, has not shown characters which would suggest the R form of culture, at least when first isolated. The colonies do not present an irregular border and do not flocculate in liquid media. There is an indication, however, that the secondary growth may readily undergo partial transformation to the rough type which flocculates spontaneously and yields an irregular colony.

d'Hérelle[3] has intimated that all fixed bacterial mutations are produced through the action of bacteriophage. The changes which occur in the colonies of the scours organism do bear a superficial resemblance to changes found in the colonies of susceptible bacteria exposed to bacteriophagic action. The fact that they regularly recur in generation after generation is also suggestive of the influence of some transmissible agent, carried on in subcultures or generated anew with each transfer. Unlike the eroded areas of lytic colonies, however, the indentations of the scours growth tend to increase in size as the colony ages. Moreover they are sometimes delayed until the colony is relatively old. With such a relationship in view a typical strain of the scours organism has been examined for evidence which might indicate the presence of bacteriophage.

Attempts to demonstrate a transmissible principle in cultures of the primary, mucoid type, using lysis as an indicator, have regularly met with failure. Filtrates made from 6, 24, and 48 hour bouillon cultures of a typical mucoid strain, *B. coli* 223 A, possessed no demonstrable lytic action.

In practice, 1:10 and 1:100 dilutions in 5 cc. of bouillon were made from the filtrates and inoculated with 0.05 cc. amounts of 18 hour bouillon cultures picked from individual colonies of the homologous type. At 37°C. growth was normal macroscopically and microscopically through 48 hours. Plates streaked from 1:10 dilutions after 6 hours of incubation showed normal colonies comparable to those of a control plate streaked from a plain bouillon culture. Successive passage of

[3] d'Hérelle, F., The bacteriophage and its behavior, translated by Smith, G. H., Baltimore, 1926.

the mucoid type through a filtrate likewise failed to elicit any demonstrable lytic action. The filtrate of a 48 hour bouillon culture of *B. coli* 223 A, diluted 1:10 with bouillon, was inoculated with 0.05 cc. of an 18 hour culture of the same type. After 48 hours at 37°C. the culture was filtered, the filtrate again diluted 1:10, and reinoculated. This procedure was continued for six passages. The final filtrate was tested, as before, by low serial dilution in bouillon seeded with equal amounts of a young culture of 223A. Growth was normal in the dilution tubes. There was no inhibition, no macroscopic alteration in the nature of the growth, and no microscopic change in the morphology of individual organisms. Plates streaked from the 1:10 dilution likewise showed normal growth. Both the original and passage filtrates were also inactive for the non-mucoid variant.

The association of a bacteriophage with the non-mucoid variant seemed less probable. However, 24 and 48 hour filtrates of the variant and later a single filtrate subjected to serial passage were tested in low dilution against the homologous type and also against the mucoid type. Growth was followed at intervals through 48 hours and plates were streaked after 6 hours. No inhibition of growth and no change in the character of growth were observed with either type. There was no indication that the variant, non-mucoid form of *B. coli* 223 was lysogenic.

The exposure of a susceptible organism to bacteriophagic action is frequently followed by the appearance of a secondary growth which is relatively resistant to lysis. The variant form of the organism under discussion might represent such a secondary growth arising in response to the action of a lytic agent. If such were the case it should possess some degree of resistance to bacteriophagic action while the parent, mucoid form should be susceptible. An effort was made to recover an active lytic agent, from the animal host, as an indicator of the susceptibility of the two culture types.

Fecal samples were obtained by swab from a series of ten calves. Two individuals, Nos. 3 and 4, were scouring when the samples were taken. The remainder were recovered cases from which feces were secured within a week following the disappearance of active symptoms. Heavy suspensions were made from the swabs in approximately 15 cc. of bouillon and incubated at 37°C. After 3 to 6 hours an agar plate was streaked from each culture. After 48 hours they were filtered through paper and a Berkefeld N candle. The filtrates were tested for lytic activity against the A and E types of *B. coli* 223 and against at least one strain, presumptively identified as *B. coli*, isolated from the corresponding plate. Growth was observed in 1:10 and 1:100 bouillon dilutions of each filtrate and on

agar plates streaked from them. The activity of the individual filtrates against the three strains is given in Table I.

Only one sample from each calf was examined and a second passage of the cultures was not attempted with individual filtrates which showed no initial activity. Hence the results indicate only roughly the distribution of bacteriophage within the series. The filtrate from Calf 1 was selected for further study. Preliminary examination had failed to indicate any difference in the strength of the two filtrates which were active for the scours type.

TABLE I.

Action of Calf Filtrates on B. coli Strains.

Calf No.	Type of *B. coli*		
	223 A	223 E	Fecal culture
1	−	+	+
2	−	−	−
3	−	−	+
4	−	−	+
5	−	−	+
6	−	−	−
7	−	−	−
8	−	−	+
9	−	+	−
10	−	−	−

The above filtrate was retested against the non-mucoid E type of *B. coli* 223. Dilutions ranging from 10^{-1} through 10^{-12} were made in 5 cc. amounts of bouillon and inoculated with 0.05 cc. of an 18 hour bouillon culture. A culture control was included. Separate pipettes were employed throughout for each mixing. After 1 hour at 37°C. there was a faint, diffuse turbidity in all tubes. Microscopically, by hanging drop, the first two dilutions showed numerous small clumps together with motile free forms. After 2 hours there was a fine, macroscopic flocculation in the first two tubes. Microscopically there was an increase in the size and number of clumps. In addition, many individual bacteria, both clumped and free, were definitely increased in size. After 6 hours the supernatants of the first two dilutions were nearly clear, a few floccules remaining suspended. There was an abundant granular sediment at the base. The third dilution showed no visible flocculation but a scant granular sediment. The turbidity of the remaining dilutions and control was diffuse, with no floccules and no sediment. Microscopically the first two dilutions, after shaking, showed clumps of varying size with

comparatively few free forms. The majority of the bacteria displayed a swollen appearance with an increase in size of from 2 to 5 or more diameters. There was a marked variation in the shape of individual cells. Spherical, elliptical, and drumstick shapes were most common. The larger forms frequently showed one or more central vacuole-like areas of different refraction from the remainder of the cell. In addition, indistinct granules were sometimes visible. The larger of the swollen forms bore a resemblance to yeast cells. Decreasing numbers of these forms and clumps were seen through dilution 10^{-5}. The remaining dilutions and the control showed no swollen forms and only an occasional small clump.

After 24 hours there was a noticeable decrease in the bulk of the sediment at the base of the first two dilution tubes, which could not be attributed simply to gravity. The supernatants showed a faint turbidity with floccules in suspension. Dilution 10^{-3} displayed a heavier sediment with a turbidity distinctly less than that of the control. With dilutions 10^{-4}, 10^{-5}, and 10^{-6} the turbidity was equal to that of the control, but there was a scant granular sediment with floccules suspended in the lower third. The remaining dilutions and the control showed a heavy, diffuse turbidity and a very scant, compact, button-like deposit quite different from that of the preceding dilutions. Microscopically the first two dilutions showed a marked decrease in the number of swollen forms. Clumps were still visible. Some showed sharply outlined bacteria but in many cases the individual units appeared indistinct, often granular in shape. Dilutions 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} showed many clumps together with free normal bacteria and an occasional swollen form. The bacteria in the remaining dilutions and the control were evenly distributed in the fields with a few small clumps and no swollen forms.

After 48 hours there was no appreciable change in the macroscopic or microscopic appearance of the first six dilutions. Dilutions 10^{-7} and 10^{-8} were slightly less turbid than the control with scant granular sediment and suspended floccules in the lower portions. The remaining dilutions and the control showed comparable turbidity with no flocculation and no change in the sediment. Dilutions 10^{-1}, 10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11} were filtered through Berkefeld candles and retested in one dilution only, 10^{-1}. The four highest dilutions showed normal growth macroscopically and microscopically through 48 hours at 37°C. Dilution 10^{-8} showed a few clumps and small swollen forms after 6 hours. After 24 hours there was a decreased turbidity with granular sediment and suspended floccules. The 10^{-1} dilution faithfully reproduced the original reaction.

A portion of the same filtrate was likewise tested against the mucoid A type, of *B. coli* 223. The same method was employed but only five dilutions from 10^{-1} to 10^{-6} were included. The tubes were examined at the same intervals as before through 48 hours at 37°C. Growth was normal in all dilutions from the start and at no time showed any variation from that of the control. Swollen forms were not seen in hanging drop preparations. Plates streaked from the 10^{-1} dilution and from the control showed no significant difference in the number of colonies and no deviation from normal in their appearance. Since the original filtrate displayed no initial lytic activity for the mucoid type an attempt was made

to adapt it. Six passages of the mucoid type were carried out with 48 hour intervals between filtrations. The final filtrate showed no demonstrable lytic action against the passage type of the organism when tested as before.

Serial passage was also resorted to in attempts to increase the activity of the original filtrate for the non-mucoid E type. Six passages were made with 48 hour intervals between inoculations. The final filtrate was tested in a dilution series with a range from 10^{-1} to 10^{-12}. The lytic strength of the filtrate was not appreciably affected. It displayed no greater tendency to cause complete lysis of the homologous bacteria, with permanent inhibition of growth, than did the original filtrate. There was, however, an increase in titer. A marked reaction was shown in dilution 10^{-3} after 6 hours and a scant reaction in dilutions 10^{-9} and 10^{-10} after 48 hours. The limiting dilution was determined, as before, by filtering and retesting in low dilution after 48 hours. Fourteen additional passages were carried out with a 24 hour interval between inoculations. The final filtrate showed no significant difference in action or in titer from the previous one. Like the original filtrate it was inactive for the parent, mucoid type of culture.

DISCUSSION.

The activity of the filtrate employed in the preceding work was weak but nevertheless definite. A growing culture of the non-mucoid variant upon exposure to a low dilution of the filtrate was first agglutinated and then altered morphologically. Individual cells became markedly exaggerated in shape, size, and internal structure. By prolonged examination it was at times possible to observe the rupture of the swollen cells. There was immediate shrinkage, with a minute granule-like residue. Complete dissolution apparently did not occur in most instances. Hanging drop preparations made from 24 hour filtrate cultures showed clumps composed in part of minute, poorly staining granules, together with an occasional swollen form and sometimes clearly outlined, deeply staining rods. The factor accountable, in part or in whole, for the cellular agglutination was slightly active for bacteria killed by heat. With dead bacteria, however, the agglutination was slow, occurred only in low dilutions, and was not accompanied by any morphological alteration of the cells. The active principle of the filtrate was relatively sensitive to heat. Exposure of the undiluted filtrate to a temperature of 55°C. for 30 minutes resulted in partial inactivation. At 65°C. for 30 minutes there was complete inactivation.

The weak lytic activity of the filtrate was demonstrable only in the

presence of the non-mucoid variant type of culture. In the presence of the parent, mucoid type it was inactive. The anomalous susceptibility of the two forms of growth together with the inactivity of their filtrates is opposed to the natural association of bacteriophage with the described scours organism as the agent responsible for variation.

CONCLUSIONS.

1. A lytic agent was not demonstrable in culture filtrates of either the parent or variant type of the scours organism.

2. The parent type was resistant to the action of a "weak" bacteriophage, obtained from the animal host, while the variant type was susceptible.

3. Exposure of the variant type to the scours bacteriophage was attended by agglutination of the cells, marked swelling, and an alteration of the contents prior to lysis.

4. The manifestations of variation which regularly occur on agar plate cultures of the scours organism do not appear to be the result of bacteriophagic stimulation.

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE.

XII. The Effect of Diet on Host Resistance.

Further Studies.

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(Received for publication, May 31, 1927.)

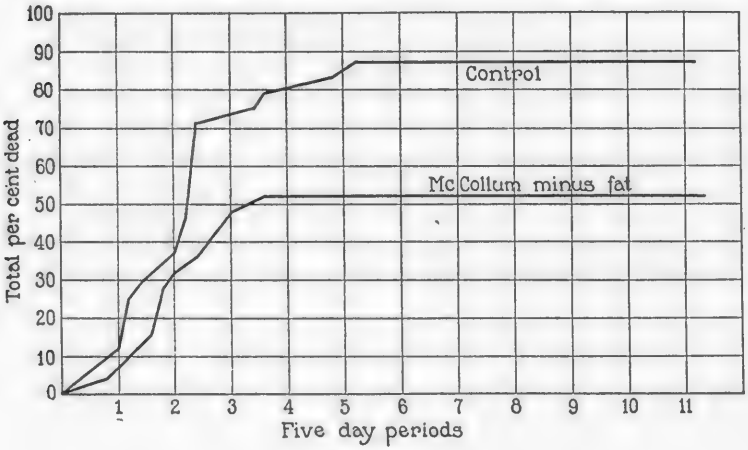
In a previous paper we described experiments in which it was shown that mice of the Rockefeller Institute strain, when fed on a McCollum "complete" diet, were far more resistant to *per os* infection with a mouse paratyphoid bacillus than were mice of the same strain fed on the routine bread and milk diet.[1] Since this paper was published, we have had it in mind to analyze the results in order to find out, if possible, which constituents of the McCollum diet were chiefly responsible for the increased resistance to infection. This work has been done during the past year, and we have sought to ascertain (1) the constituents of the McCollum diet which promote resistance to the infection, and (2) whether the observed seasonal fluctuations in resistance to the infection[2,3] are correlated with seasonal changes in diet.

The first experiments were planned to determine (*a*) the effect of the omission of the butter fat from the McCollum diet, and (*b*) the effect of the constituents of that diet, other than butter fat, when added singly to the ordinary bread and milk ration.

The mice employed were obtained from the Rockefeller Institute breeding room and had been reared on a diet of bread and milk, grain mixture, and dog biscuit,

[1] Webster, L. T., and Pritchett, I. W., *J. Exp. Med.*, 1924, xl, 397.
[2] Pritchett, I. W., *J. Exp. Med.*, 1925, xli, 209.
[3] Pritchett, I. W., *J. Exp. Med.*, 1926, xliii, 173.

from the time of weaning until they were 6 to 8 weeks of age. They were then removed to another building and placed in metal cages in groups of 12 and 13. At this time the various modified diet groups were arranged, a control group being maintained on the ordinary bread and milk diet. The mice were given the same care that they received in the breedng room; the cages were kept as clean as possible, uneaten portions of the ration being removed at the end of the day. On the day of inoculation each mouse was placed in a separate battery jar containing clean shavings and received by means of a stomach tube about 5,000,000 bacilli from an 18 hour broth culture of mouse typhoid (*B. pestis caviæ*). Each test was allowed to run 8 weeks.



TEXT-FIG. 1.

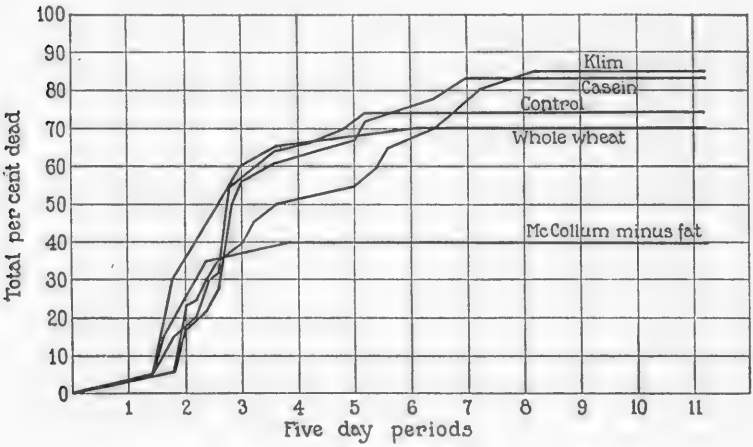
EXPERIMENTAL.

Experiment 1.—Two groups of mice of the Institute strain, one lot of 24 and another of 25, were caged in groups of 12 and 13. One lot, consisting of 25 mice, received, in addition to a constant supply of fresh water, a McCollum diet from which the 5 per cent of butter fat had been omitted, the rest of the formula being the same as that previously employed:

Whole wheat flour	67.5 per cent.
Casein (commercial)	15.0 " "
Milk powder (Klim)	10.0 " "
Sodium chloride	1.0 " "
Calcium carbonate	1.5 " "

The second lot, consisting of 24 mice, was fed on the bread and milk diet and served as controls. After having been on these diets for a period of 10 days, each mouse was given by means of a stomach tube the standard dose of about 5,000,000 mouse typhoid bacilli.

The mortality curves for these tests are given in Text-fig. 1. From them it will be seen that the death rate of the McCollum group is lower than that of the bread and milk group, though not so low as were the rates previously observed when the complete McCollum formula, including the butter fat, was fed.[1]



TEXT-FIG. 2.

Experiment 2.—This test was planned to control Experiment 1 and in addition to try the effect of the various constituents of the McCollum diet, other than butter fat, when added separately to the routine bread and milk diet. The following diet groups were arranged, the substances used in the modified diets being thoroughly mixed with the bread and milk ration before feeding.

A. 20 Institute mice. Bread and milk diet plus 10 per cent powdered whole milk (Klim).
B. 18 Institute mice. Bread and milk diet plus 10 per cent commercial casein.
C. 20 " " " " " " " " 10 " " whole wheat flour.
D. 47 " " " " " " " only—controls.
E. 20 " " McCollum diet minus butter fat.

The mice were kept as before in small groups in cages. After 2 weeks on the various modified diets, each mouse was placed in a separate battery jar and re-

received *per os* the usual standard dose of mouse typhoid bacilli. The results of this experiment are given in Text-fig. 2.

It will be seen from Text-fig. 2 that the mortality rate in all groups, except the modified McCollum diet group, closely approximated that of the controls. Thus none of the constituents of the McCollum diet that were tested separately in this experiment had been capable of conferring a resistance equal to that of a combination of the same substances, to which was added a salt mixture.

It appeared from this experiment that Klim, casein, and whole wheat flour exerted by themselves little beneficial effect upon the mice. A third experiment was therefore planned to test the effect of the addition of butter fat, the chief remaining constituent of the McCollum diet, to the control bread and milk diet. Cod liver oil, a substance of somewhat similar dietary properties, was also tested.

Experiment 3.—Mice of the Institute strain were brought up from the breeding room on December 1, 1925. The following diet groups were arranged:

A. 22 Institute mice. Bread and milk diet plus 10 per cent butter fat.

B. 22 " " " " " " " 10 " " cod liver oil (Harris).
C. 24 " " " " " " only—controls.
D. 24 " " McCollum diet minus fat.

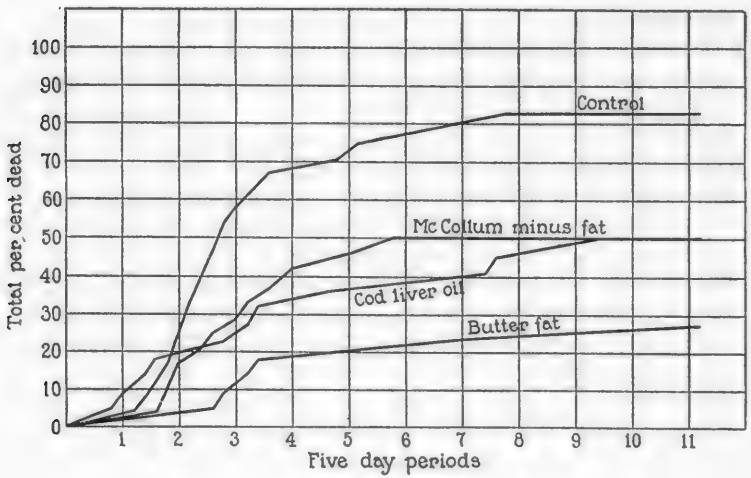
On December 15, each mouse received *per os* the usual standard dose of mouse typhoid bacilli, containing about 4,250,000 organisms. The results of this experiment are given in Text-fig. 3.

It will be seen from Text-fig. 3 that the mortality rates of all the groups on the modified diets were considerably lower than that of the control group. The inference drawn from these experiments is that butter fat is the most important single constituent of the McCollum "complete" diet in raising the resistance of mice of the Institute strain to infection with the mouse typhoid bacillus, and that the same protective action may perhaps be found in cod liver oil.

A series of experiments was now performed in an effort to determine the relative efficacy of various fat-containing substances in protecting our mice against experimental mouse typhoid infection. Keeping in mind the possibility, already mentioned, that observed seasonal fluctuations in resistance might be due in some way to seasonal

changes in diet, we employed for these experiments (*a*) a fat known to vary seasonally in its content of accessory food factors, (*b*) a fat known to be relatively constant in its content of such factors, and (*c*) a fat thought to be free of vitamins. They were represented by the following fats and oils:

1. Butter fat (from Borden's sweet butter). Subject to seasonal fluctuations in its content of fat-soluble vitamin.
2. Cod liver oil (Harris). A concentrated and relatively uniform source of fat-soluble vitamin.
3. Crisco. A hydrogenated vegetable oil thought to be lacking in vitamins.



TEXT-FIG. 3.

In addition two other diets were employed:

4. A bread and milk diet in which the milk had been subjected to the direct light of a small mercury vapor lamp, for 1 hour.
5. McCollum diet minus fat.

Experiments with several of these diets were carried out throughout the first half of the year 1926, from January to June inclusive, since previous studies had indicated that at this time of year our mortality rates were likely to be at their highest.[2,3] The milk employed

TABLE

Experiment started	Diet	Number of mice	Number of mice															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1.15.26	Bread and milk + 10% butter fat	25									2	2	1	2	1			1	
	McCollum minus butter fat	24				1		1			3	1	1	1	1			2	
	Bread and milk—control	24						1			3	1	1	4	1	1		1	2
2.16.26	Bread and milk + 5% butter fat	20									1	2	2		3				
	Bread and milk + 5% cod liver oil	20								1		2	1						2
	Bread and milk + 5% Crisco	20									2		1	1	2			2	
	McCollum minus butter fat	20					1				2	1	1				1		1
	Bread and milk—control	18									1	3	2	2	3	1			
3.16.26	Bread and milk + 5% butter fat	30				2		1	1		1	1			2	1	2	1	
	Bread and milk + 5% cod liver oil	29						1	2	2	3								
	Bread and milk + 5% Crisco	29				1			1	4	1	1			1			1	1
	Bread and milk—control	31					1				2	1	2	1	4	1			1
4.15.26	Bread and milk + 5% butter fat	29						1		1	1	3	2	1	2	1	1		
	Bread and milk + 5% cod liver oil	30						1			3	1	2	1					1
	Bread and milk + 5% Crisco	29									1		3			2			1
	Bread with rayed milk	30								2	2	2	1	1				1	
	Bread and milk—control	30					2			2	1	2	2	4		1		2	1
5.14.26	Bread and milk + 5% butter fat	29							1		2	2		2					
	Bread and milk + 5% cod liver oil	30						2	1	2	1	1	2						
	Bread and milk + 5% Crisco	30					1			2	2	4	2	2	2	2		1	
	Bread with rayed milk	30							1		2	2	1			1			
	Bread and milk—control	30							1	3	1	1	1	1	2	2	1		
6.15.26	Bread and milk + 5% butter fat	30							1	2	1		1	1	1	2		2	
	Bread and milk + 5% cod liver oil	30							1	3	1	1	2	1		1	1		
	Bread and milk + 5% Crisco	29							1	2		1		2	1	1	2		
	Bread with rayed milk	30								2	2	2				2	3		
	Bread and milk—control	30								2	2	2		1		2			1
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17

L

| dying each day | | |

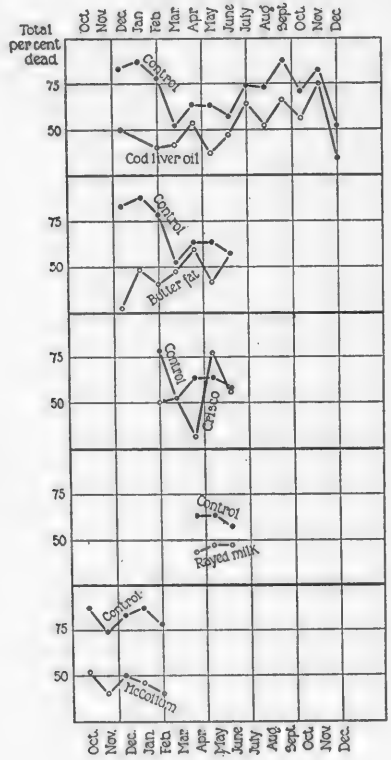
TABLE I

Experiment started	Diet	Number of mice	Number of mice															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
7.27.26	Bread and milk + 5% cod liver oil	50							1	2	2	1	3	3	1	1	2	3	
	Bread and milk—control	50							1	2	2	2	1	3	1		3		1
8.27.26	Bread and milk + 5% cod liver oil	50								1	3		4	1	5	1			1
	Bread and milk—control	48						1	2	2	1	2	3	1	6	3			
9.22.26	Bread and milk + 5% cod liver oil	50						1		4	1	5	2	3	2				
	Bread and milk—control	50						2	1	1	3	7		16	3		1	1	
10.20.26	Bread and milk + 5% cod liver oil	48						1	2		5	3		1	4		2	1	
	Bread and milk—control	48					2	1			1		1	2	5		3		
11.16.26	Bread and milk + 5% cod liver oil	48							1	2	3	2	1	2	7	6			1
	Bread and milk—control	49						3	1		3	2	5	4	15	3	2		1
12.15.26	Bread and milk + 5% cod liver oil	50								1		2	2			3		1	
	Bread and milk—control	50									3	1	7	1	1	1	2	1	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17

—*Concluded.*

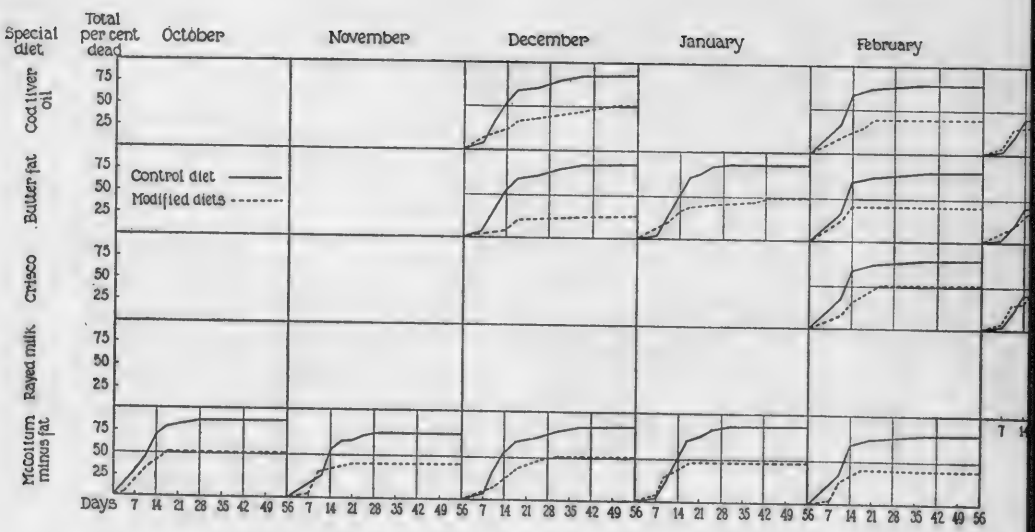
dying each day																																											Total dead	
18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	Number	Per cent
1	1				2	1	1	1		3							1						1	1																	32	64
				1		1		2			2	1				6						1	3											1			1			37	74
	1					1													1					2		1				1						1		1	26	52
		7				1							1										1	2						1							1			35	73
3	3	1	3				1			1	1										1		1																	33	66
	1	2				1	1					2							1				1																	44	88
	3					1		1														1				1				1										27	56
1	4	1	1					2	1	1					3							2	2								1									34	71
		2	1	1	1	1			1	2			1		1																									36	75
																					1																			40	82
	1			1			1								2													2			1								17	34
	1											1					1										4	1						1						26	52
8	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56		

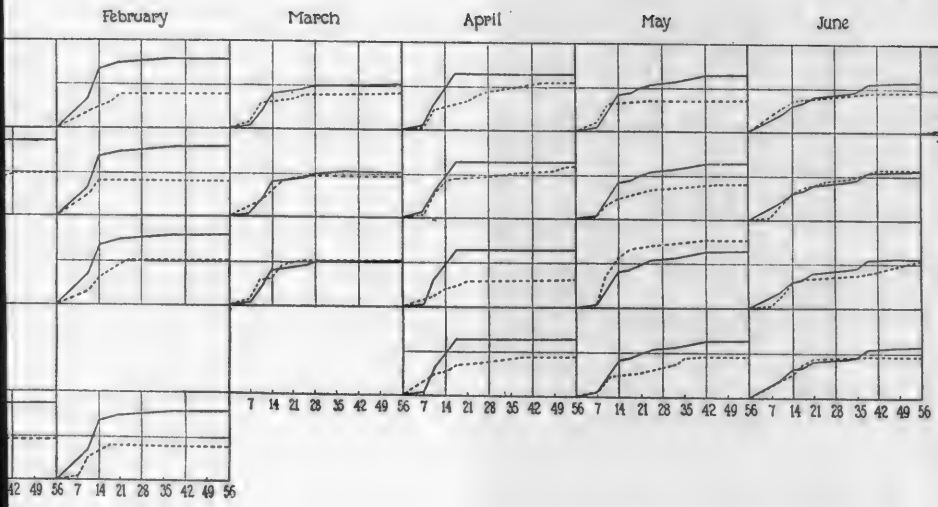
for Diet 4 above was identical with that used for the control bread and milk diet except that it had been exposed for 1 hour, in a shallow glass vessel, at a distance of 4 or 5 inches, to the light of a small mercury vapor lamp, and was thoroughly stirred with a pipette at the end of the first half-hour. From the month of March on the modified Mc-



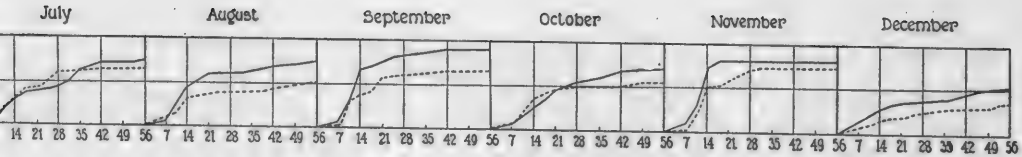
TEXT-FIG. 4.

Collum diet (minus butter fat) was abandoned, and only the bread and milk control diet together with its fat and rayed milk variants was employed. This was done in an effort to simplify the problem as much as possible and to permit the use of larger numbers of mice in each diet group. From February on only 5 per cent of fat was used in the


Special diet
Total per cent dead
October
November
December
January
February
Cod liver oil
Butter fat
Crisco
Rayed milk
McCollum minus fat
75
50
25
Control diet
Modified diets
Days
7 14 21 28 35 42 49 56



TEXT-FIG. 5.


July
August
September
October
November
December
14 21 28 35 42 49 56 7 14 21 28 35 42 49 56 7 14 21 28 35 42 49 56 7 14 21 28 35 42 49 56 7 14 21 28 35 42 49 56 7 14 21 28 35 42 49 56

modified diets, instead of the 10 per cent employed in the earlier experiments. This was done in order that the fat content of the modified bread and milk diets might be more nearly comparable to that of the whole McCollum diet, the original formula for which, as given us by Dr. E. V. McCollum, called for 5 per cent of butter fat. The results of all these experiments are condensed in Table I and in Text-figs. 4 and 5.

Text-fig. 4 shows the monthly mortality rate for each of the modified diet groups, and for the corresponding control groups, from October, 1925, to December, 1926, inclusive, thereby including all the experiments recorded in this paper. The mortality rate for any one month represents the total mortality of the mice used in the experiment begun in that month, all experiments being started on or near the 15th. Although the points on this chart are discontinuous, related points have been roughly connected into curves, in order to enable the reader to compare more easily the mortality rates of the mice on the various modified diets with the rate of the controls on the plain bread and milk diet. The total per cent mortality is indicated along the ordinate, while the successive months are arranged along the abscissa. In each division of the chart appear two curves—that of the diet group indicated, together with that of the control group for the same period. In October and November only the McCollum diet minus fat was used, in addition to the control bread and milk diet. From December on, several fat diets were included in the experiment and for each one a separate curve is plotted, in each case accompanied by the corresponding portion of the control curve. These diets were continued through June, 1926, after which time only the cod liver oil and the control diet groups were employed.

It will be seen that, almost without exception, the total mortality rate of the control group was higher than that of the groups on the various modified diets. The results presented here are somewhat fragmentary, since only the cod liver oil and control series were carried on throughout the year. Nevertheless, within the longer or shorter periods covered, the effects of all the modified diet groups, save one, were consistent. In every case except that of the group receiving the bread and milk diet plus 5 per cent Crisco, the mortality rate is consistently lower than in the control group on the unmodified bread and milk diet. They follow with considerable regularity the fluctuations

in mortality of the control group, a tendency to stabilization of the death rate at a level lower than that of the controls being indicated. The mortality rate of the Crisco series, on the other hand, was three times lower than, once higher than, and once equal to that of the control series.

Text-fig. 5, like Text-fig. 4, is compiled from the data given in Table I, and shows the separate mortality curves of each of the monthly diet experiments. In each division of the chart appear two curves, plotted as total mortality against time—that of the diet group indicated along the left margin of the chart together with the corresponding control curve. The curve for the control group in any one month is therefore repeated as many times as there were modified diet groups in the experiment for that month. In this chart it is easy to compare the separate diet curves throughout their course with the corresponding control curves. It will be seen that for the most part the various diet curves exhibit considerable similarity in their tendency to approximate or diverge from the corresponding control curve in any given month. In December, January, and February, the final mortality rate of all the modified diet groups was considerably below that of the controls. In March, however, the mortality rate in the control group dropped and closely approached the level usually attained by the groups on the modified diets. In April and May, the death rate in the control group increased somewhat and again diverged from the level usually attained by the modified diet groups; the only decided exception is in the May group receiving the modified diet plus Crisco, which showed a mortality rate above that of the May control group. In June, the mortality rate in the control group again dropped and approximated the level maintained, with fair regularity, by the modified diet groups.

In the period from July to December inclusive, only the control diet and the cod liver oil diet were employed, 50 mice being included monthly in each diet group. In July, both curves were surprisingly high for this time of year and closely approximated each other. Thereafter they showed the customary divergence, except in the month of November, when both were again high.

It seems to be clearly indicated by these experiments that butter fat is the most important single constituent of the McCollum diet in

protecting the mice against *per os* infection with the bacillus of mouse typhoid. The degree of protection afforded by butter fat, when added to the control diet, was about equal to that of all the other constituents of the McCollum diet combined, and was closely approximated by that of cod liver oil.

While seasonal fluctuations in resistance were not completely eliminated by the various modified diets, they were reduced. There was apparently a tendency for the various modified diets to stabilize the death rate at a point lower than that usually reached by the mice on the control diet.

DISCUSSION.

Recently a paper has appeared by Vaile[4] on the correlation between fat consumption and susceptibility to tuberculosis. Through a period of 7 years' country practice in England, he studied various families in which cases of tuberculosis had occurred, dividing up their members into two groups according to whether they were or were not normal fat eaters. He concluded that "when several persons are equally exposed to infection [with tuberculosis], those who habitually eat animal fat will probably escape—but may not, while those who avoid fat may escape—but probably will not." He could not see that forcing fat consumption above the level reached by the normal fat eater was profitable.

So far as I am aware, most of the published experiments on the effect of diet upon susceptibility to disease are concerned primarily with diets in which one or more of the accessory food substances is markedly deficient or wholly lacking. In the experiments here recorded, this was not the case. The "control" diet employed here was one which had been shown, through a period of years, to be adequate for the breeding and rearing of successive generations of mice, apparently without any loss of vigor. Though somewhat fragmentary, the experiments here recorded do suggest, however, that the addition of 5 per cent of an active animal fat to an apparently adequate diet can so improve its quality as to increase the resistance of mice fed on such a modified diet to *per os* infection with the bacillus of mouse typhoid. The reason for the beneficial effect of these modifications of our bread

[4] Vaile, W. B., *Lancet*, 1927, ccxii, 72.

and milk diet, aside from the probable promotion of better general health, is not known. The bread and milk food may be one of the borderline diets referred to by Cramer,[5] who observes that the deficiencies of an apparently adequate diet may manifest themselves only when the animal is subjected to a strain. It is difficult to say whether these deficiencies could be made good in a scant 2 weeks' feeding on the modified diets, before inoculation with the bacillus of mouse typhoid. The consistency of the results would seem to indicate, however, that the apparently increased resistance of the mice on the modified bread and milk diets containing butter fat and cod liver oil, as well as those on the modified McCollum diet, was not due to chance.

SUMMARY.

When 5 per cent of butter fat or cod liver oil is added to a bread and milk diet, in itself adequate to promote the breeding and rearing of a healthy stock of mice through a period of years, the resistance of these mice to *per os* infection with the paratyphoid mouse typhoid bacillus (*B. pestis caviæ*), as compared to that of mice on the unmodified diet, is definitely increased. A similar effect may be obtained with a McCollum "complete" diet, even when the butter fat is omitted, and with a bread and milk diet in which the milk used has been rayed with a mercury vapor lamp. When an inactive fat like Crisco is added to the bread and milk diet, the results obtained are not very clear-cut. While seasonal fluctuations in resistance to mouse typhoid were not completely eliminated by the various modified diets, they were nevertheless reduced, the modified diets tending to stabilize the death rate at a point lower than that usually reached by the mice on the control diet.

[5] Cramer, W., *Brit. J. Exp. Path.*, 1922, iii, 298.

AN EXPERIMENTAL STUDY OF DIATHERMY.

I. The Measurement of Lung Temperature.

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(Received for publication, June 20, 1927.)

INTRODUCTION.

The word "diathermy" means heating through. It was coined by Nagelschmidt (1) to describe the effects of the passage of certain electrical currents through the human body. Tesla[1] (2) is responsible for perfecting apparatus whereby high tension alternating currents of high frequency can be produced. In the *Electrical Engineer* for December, 1891, Tesla says: "Without vouching for all the results, which must of course be determined by experience and observation, I can at least warrant the fact that heating would occur by the use of this method of subjecting the human body to bombardment of alternating currents of high potential and high frequency." He goes on to throw out the suggestion that such heating of the body may some day find an important place in therapeutics. This prediction is in a sense correct. Diathermy has come to be one of the most widely used of the so called "physiotherapeutic modalities." Moreover, a copious literature, mostly of an empirical and casuistic kind, has sprung up, dealing with the therapeutic application of diathermy in a variety of clinical conditions. Much of this prolific literature attributes the beneficent action of these currents solely to the production of local deep heat, though some writers go as far as to stress the value of "cellular massage," which they claim the current produces. Medical diathermy, as distinguished from fulguration and cauterization, is today being used in the treatment of many diseases,

[1] We have since learned that Dr. Elihu Thomson of the General Electric Company was probably the first to construct a dynamo capable of producing high frequency currents.

such as angina pectoris, arteriosclerosis, arthritis, asthma, bronchitis and bronchiectasis, cholecystitis, essential hypertension, gonorrhea, gout, hemorrhoids, impotence, intermittent claudication, lumbago, lupus, myocarditis, neuralgia, paresthesias, pneumonia, Raynaud's disease, scleroderma, sciatica, tabes, tuberculosis, uterine displacements, varicose veins, whooping cough, and xanthelasma. For the successful treatment of each one of these conditions there are one or more enthusiastic champions.

Such a list naturally gives one pause and makes for skepticism, especially as it is so extraordinarily difficult to arrive at sound judgments in regard to remedial measures. Still, it should be borne in mind that the application of heat with the production of hyperemia is a time-honored medical procedure and one which may well have unsuspected usefulness. In the treatment of pneumonia, particularly, reports of the successful application of diathermy have been numerous, widespread, and enthusiastic. Stewart's (3) work is especially suggestive. The rationale for the use of diathermy in pneumonia has been variously ascribed to the hastening of resolution, the stimulation of the normal defenses of the body, and the direct lethal action on the microorganisms of the heat developed in the lung.

We were led to a study of the bodily responses to high frequency currents as a preliminary to an investigation of the value of diathermy in pneumonia. D'Arsonval (4) made the fundamental observation that when this type of current is passed through the human body muscle contraction is felt up to about 5000 interruptions per second. At 5000 interruptions muscle contraction is feeble, disappearing beyond 10,000.[2] At these frequencies, however, there occurs an unpleasant feeling of heat. The problem which confronted us was to find out whether deep localized heat could actually be produced in the body. Before presenting an account of our experiments and the

[2] Nernst (5) developed mathematically a generalization stating that the physiological threshold for relatively weak alternating currents varies inversely as the square root of the frequency. The number of times a current must alternate if it is to lose its stimulating and electrolytic powers depends upon its strength. Strong currents must have a higher frequency than weak ones. The Nernst formula is $I = \sqrt{NC}$, where I = intensity of alternating current, N = number of complete cycles per unit of time, and C = a constant.

results obtained from them, it will be well to consider briefly the character of the so called diathermy current and some of the laws governing its use.

The Nature of the So Called Diathermy Current.

The diathermy current is an alternating current of high voltage and low amperage, very rapidly reversed in its polarity. Usually the voltage employed in the secondary circuit is approximately 30,000 to 40,000, the voltage in the body being lower, but not accurately ascertainable. The current is from 1 to 2 amperes, and the frequency of oscillation about 1,000,000 to 2,000,000 cycles per second.[3] Such currents can be produced by suitable apparatus consisting essentially of the parts shown in Fig. 1.

In this diagram *b* is the core of a transformer which transforms the low voltage current in the primary coil (*a*) into a high voltage current of the same frequency in the secondary coil (*c*). This current charges the condenser (*d*) to a voltage sufficient to break down the insulation of the spark-gap (*e*). The condenser (*d*) then discharges through the circuit *d e f* with an alternating current of high frequency determined by the small capacity of (*d*) and the small inductance of (*f*). The current induces in the oscillator (*g*) a current of the same high frequency but of again increased voltage.

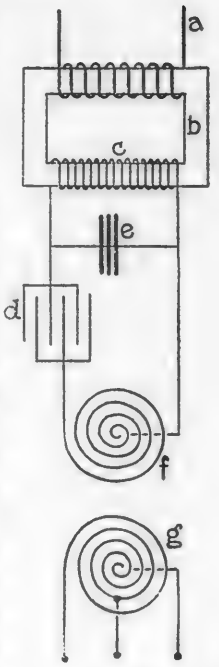

FIG. 1. Diagram of high frequency apparatus after Cumberbatch (6). (*a*) primary coil, (*b*) transformer, (*c*) secondary coil, (*d*) condenser, (*e*) spark-gap, (*f*) oscillator, (*g*) resonator.

[3] The physical constants of the apparatus which we used were:
Primary voltage, 110.
Secondary transformer voltage, 36,000.
Frequency in primary circuit, 60 cycles per second.
Frequency in secondary or oscillating circuit, 1,250,000 cycles per second.
Amount of capacity in secondary circuit about 900 cm.
Damping factor in secondary circuit, or logarithmic decrement, slightly below 0.5.

It is not unlikely that modern radio equipment may soon be used to advantage to replace this type of spark-gap machine.

The frequency of oscillatory discharge will depend upon: (1) the amount of self-induction in the spiral oscillator, (2) the capacity of the condenser, and, to a slight extent (3) the resistance of the circuit.

When a current traverses a conductor, heat is generated. The number of units of heat developed in the conductor is proportional to (1) its ohmic resistance, (2) the square of the current strength, (3) the duration of flow. For a continuous current the heating effect is proportional to I^2R, where I is the steady current and R the normal resistance of the conductor at any definite temperature. With diathermy high frequency currents the heat is again proportional to I^2R, but in this instance I denotes the average flow of current throughout the successive cycles, and R is the ohmic resistance plus the effect of dielectric loss.

The heating effects of these currents have for the most part been studied in non-living systems. A favorite experiment has been the coagulation of egg albumin or the cooking of meat and potatoes. Two statements are frequently made concerning the diathermy current, first, that the current always flows through the shortest path between the electrodes regardless of the resistance it encounters; second, that the heating occurs first at the center of the current path and later at the two ends. In another communication (7) we will present evidence to show that neither of these statements is strictly accurate.

Arguments from analogy based on such *in vitro* experiments have been carried over to the clinical application of diathermy, and have been responsible for many false assumptions. The living body is not a sausage, nor yet a tube filled with albumin water. It is a heterogeneous system composed of tissues with different specific conductivities and heat capacities. Moreover, these tissues are variously placed and must therefore lose heat at different rates, according to the efficiency of their circulation, and the opportunity afforded them for heat loss by radiation, convection, and conduction. Thus far, it has not been definitely established either that deep local heat can be produced by the diathermy current or, indeed, that the current penetrates into the deeper portions of the body.

Recently, Bethman and Crohn (8) have presented evidence which they believe indicates that the so called "skin effect" is a factor in keeping the current near the surface of the body. Moreover, these authors state that in experiments with anesthetized dogs they have "found it extremely difficult at any time to raise the systemic temperature more than a few fractions of a degree." Such has not been the case in our experiments, as will be brought out later, nor in the experiments reported by Lonergan (9). Dowse and Iredell (10), applying the formula developed by Lord Rayleigh, A. Russell, and others, "assume that no measurable skin effect will be present" in the passage of a current of 10^6 periods per second through the human body. They emphasize, further, the unreliability of attempting to measure current strength in the body accurately by means of a hot wire ammeter.

The fact of the passage of current through tissue can best be established by proof of heat development in the tissue. That this heat will be quickly dissipated, and that it may be conveyed to the tissue from adjoining structures, are two complicating conditions which need to be carefully controlled.

It has been the object of this study to learn whether or not local deep heat is developed in the living animal body by the passage of the current, and if so, under what conditions.

EXPERIMENTAL.

I. Effect of Diathermy Current on the Rectal Temperature.

There can be no doubt that the general systemic temperature as measured by a rectal thermometer can be raised by the passage of the diathermy current. This has been shown by Setzu (11), von Zeynek and his coworkers (12), and Lonergan (9), as well as frequently in our own experiments. In Table I it will be seen that in unanesthetized individuals of three different species the rectal temperature as observed by us was raised from 1–2°C. in as many hours.

In anesthetized animals (dogs) we have frequently seen a rise in rectal temperature of as much as 5–6°C. This increased heating effect must be due to inability to provide for heat loss, owing to partial paralysis of the heat-regulating mechanism by the anesthetic. Nor-

mally an animal will compensate for a moderate decrease or increase in the systemic temperature by increasing the heat loss or varying the metabolic rate. Under the influence of an anesthetic, however, this mechanism may be impaired, and the systemic temperature will fall rapidly unless heat loss be prevented (13). This phenomenon is well shown in the control periods of most of our experiments. Careful wrapping of the dog in blankets prevents heat loss to a fair degree and similarly assures a greater rise in systemic temperature during the passage of the current.

TABLE I.

Species	Milliamperes per sq. inch of electrode surface	Duration of current flow	Rise in rectal temperature
			°C.
Rabbit	100	2 hrs.	2.7
Dog	90	2 hrs. 13 min.	1.1
Man	66	1 hr. 21 min.	1.0

II. Effect of Diathermy Current on Deep Temperature.

(a) Intraabdominal Temperature.

Experiment D 8.—A male mongrel terrier was anesthetized by the intravenous injection of 2.97 gm. of barbital-sodium dissolved in physiological salt solution. The sides of the abdomen were shaved and lead-tin electrodes measuring 8 × 10 cm. were applied laterally. A mercury thermometer was inserted into the rectum, a second thermometer was placed outside of the electrical field by passing it through the abdominal wall so that its bulb lay in the abdominal cavity 5 cm. from the superior edge of the electrodes. A third thermometer was so placed that its bulb lay in the abdominal cavity at a point midway between the two centers of the parallel electrodes. A diathermy current of 1000 milliamperes (with a current density of less than 100 milliamperes per square inch) was passed through the abdomen for 1 hour. The result was a rise in rectal temperature of 1.76°C., a rise of temperature in that part of the abdomen outside the electrical field of 1.76°C., and a rise of temperature in the abdominal cavity between electrodes of 2.62°C. The dog was then suddenly killed, while the diathermy current was flowing. After death the temperature in both rectum and upper abdominal cavity continued to rise at approximately the rate previous to death, while the temperature in the midabdomen between electrodes suddenly shot up and in the next hour showed an increment of 17.73°C., or nearly seven times as great a rise as recorded during life. This latter fact is in agreement with the temperature changes observed by Lonergan

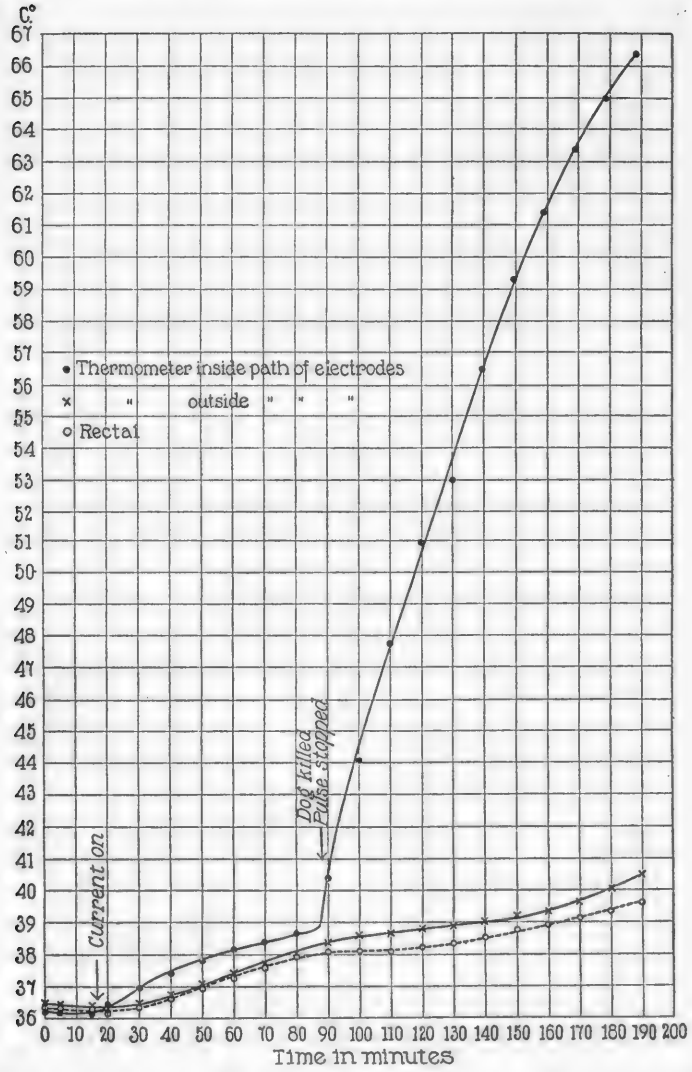

FIG. 2. Curve showing effect of death on heat recorded by thermometer placed in abdomen between electrodes.

(9) during diathermy of a dog's hip joint before and after death. The temperature changes recorded in the experiment just described (D 8) are graphically shown in Fig. 2.

This experiment brings out several points of interest. The temperature rise during life in that portion of the abdominal cavity situated between the electrodes was only a little greater than the rise in the upper abdomen and rectum. After death, however, there was a great and sudden heating up of the abdomen between the electrodes, suggesting that a compensatory cooling mechanism had ceased to operate with the cessation of life. The rectal thermometer reading here cannot be regarded as a true record of the systemic temperature because of its proximity to the heated area.

(*b*) *Intrapleural Temperature.*

In another similar experiment (D 9) in which a thermometer was inserted into the left pleural cavity between electrodes placed laterally on the shaved chest wall, the temperature during 1 hour of current flow rose by 1.88°C. with a rise in rectal temperature of 1.21°C. After death, another hour of diathermy produced an elevation in intrathoracic temperature of 11.62°C., whereas the rectal temperature increased by only 0.08°C. Death, therefore, appears to permit a local heating in the deep tissues between electrodes which does not occur during life. It seemed fairly obvious that the interruption of normal circulation would be found accountable for this discrepancy.

To study the relation of local circulation and heat production experiments were planned in which the temperature of the lungs could be measured by thermocouples. Previous work (14) had provided us with methods for modifying and interrupting the pulmonary circulation. A description of the application of these methods to the present problem will be found in a companion paper (15).

Technique for Measuring Lung Temperature.

The accurate measurement of lung temperature in the living animal during heating by diathermy was not easy to accomplish. After discovering and ruling out a number of sources of error, we achieved a method which has proved satisfactory.

The dog is anesthetized by the intravenous injection of a solution of barbital-sodium. The sides of the thorax are shaved. Lead-tin electrodes, measuring in most instances 3 × 4 inches, are applied to the chest wall with the superior edge well up in the axilla. The interposition of 8 to 12 layers of gauze soaked in a solution consisting of equal parts of glycerol and saturated salt solution assures a good contact. Great care is taken to have the planes of the electrode surfaces as nearly parallel as possible in order to prevent the so called "edge effect"—*i.e.*, the current passing largely between the proximal edges of the electrodes. A convenient method for holding the electrodes in place was accomplished by encircling the thorax with two or three rubber tubes and slipping the electrodes under them. These tubes permit the free motion of the chest wall and still, by their elasticity, hold the electrodes firmly against the skin.

Thermocouples were made by twisting together 5½ foot lengths of No. 31 gauge enamelled copper wire and double silk-covered "constantan" wire treated with one coat of shellac. These were then shellacked and allowed to dry. The two wires were threaded through the shank of a No. 20 gauge hollow Luer needle 11.5 cm. long. The exposed tips of the wires were soldered to the point of the needle in such a manner that the solder closed smoothly the bevelled opening of the needle. The wires were protected by covering them with thick walled rubber capillary tubing, the end of which was passed over the butt of the needle. The free ends of the exposed wires were connected with a galvanometer through a constant temperature junction obtained by a thermos bottle thermostat as described by Clark (16). Thermocouples such as these can be easily prepared in the laboratory. Before use they were calibrated against a Bureau of Standards thermometer which could be read to 0.02°C. When prepared from the same materials and in identical manner, the calibration curves agreed so closely that one curve could be used for all the thermocouples. The calibrations were made at known resistances and each resistance required its own curve. Resistance was supplied by an ordinary Leeds and Northrup resistance box.

The position of these thermocouple needles in respect to the electrical field was found to be of great importance. To obtain accurate temperature measurements the needles must be so placed that they cut the electrical field at right angles. In other words, they must be parallel to the planes of the electrodes, otherwise a concentration of the high frequency current occurs at the needle point, which is then heated more than the surrounding tissue. To avoid this contingency the needles were inserted into the thoracic cavity either from its anterior or posterior aspect so that the final disposition of electrodes and thermocouples was as shown in Fig. 3.

In all experiments it was made certain that turning the current on or off caused no sudden fling in the galvanometer due to concentration of current at the thermocouple. The error introduced by heat loss due to conduction through the needle shank seems a negligible one in view of the very close agreements between temperature so measured

and temperature measured by Bureau of Standards thermometers inserted in the rectum. That this source of error was negligible was shown by heating a portion of the needle shank which lay under the skin or projected outside the body wall. This caused no change in the galvanometer reading, showing that sufficient heat to cause an error in reading was not conducted along the needle to the thermocouple.

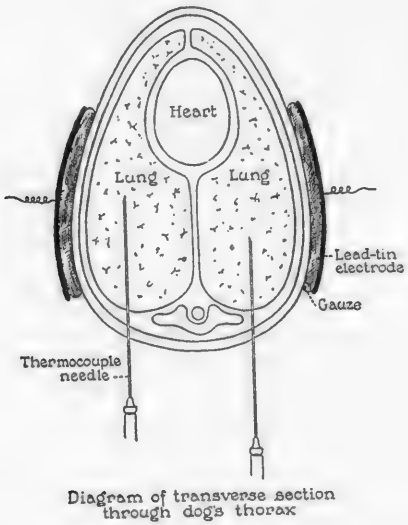

FIG. 3. Diagram of transverse section through a dog's thorax, showing relative positions of electrodes and thermocouples.

(*c*) *Intrapulmonary Temperature.*

Experiment D 12.—An experiment in which we used the general technique just described was performed on a male American bulldog weighing 14.7 kilos. Thermocouple needles were inserted into the right lower lobe, the right ventral lobe, and the left lower lobe. The position of the thermocouples was verified at the close of the experiment. Simultaneously the rectal temperature was recorded with a mercury thermometer. The average of the three lung temperature readings which agreed within ±0.05°C. before the current was turned on was 0.39°C. below the rectal temperature. During diathermy the lung temperature rose slightly above the rectal, not exceeding it by more than 0.4°C. Toward the close of the experiment,

after 3 hours of diathermy, this difference was even less. The temperatures are shown graphically in Fig. 4.

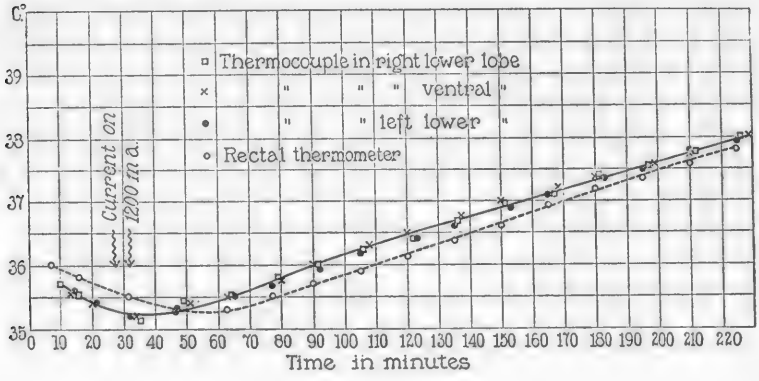
C°
Thermocouple in right lower lobe
" " " ventral "
" " " left lower "
Rectal thermometer
Current on 1200 m a.
39
38
37
36
35
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220
Time in minutes

FIG. 4.

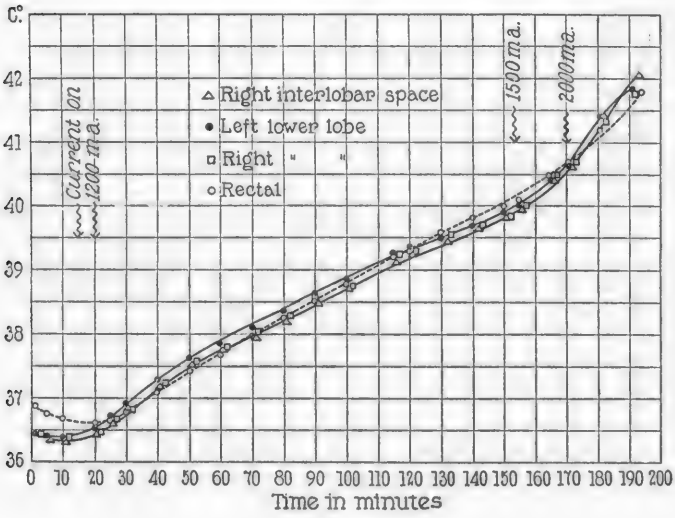

FIG. 5.

FIGS. 4 and 5. Curves showing temperatures recorded by thermocouples in the lungs and by rectal thermometers, in anesthetized dogs subjected to high frequency currents.

Experiment D 11.—This experiment was repeated on another dog. Three thermocouples were again inserted into the thoracic cavity. One of them lodged in the left lower lobe, one in the right lower lobe, and one lay between the right ventral and lower lobes in contact with the visceral pleura. The readings on these three thermocouples seldom varied by more than 0.1°C. Before turning on the current the three readings were: left lower lobe 36.45°C., right lower lobe 36.43°C., right visceral pleura 36.42°C. At this time the rectal temperature measured by a mercury thermometer was 36.88°C. After 2¾ hours of diathermy, the lung temperatures had risen respectively to 41.43°C., 41.35°C., and 41.43°C., while the rectal temperature was 41.21°C. Fig. 5 shows the lung and rectal temperatures in graphic form.

This kind of experiment has been done repeatedly, and in some cases rectal thermocouples were used in conjunction with rectal thermometers. The same type of curve was always obtained.

DISCUSSION.

Examination of Figs. 4 and 5 will show that whereas the lung temperature in the anesthetized dog normally lies 0.3–0.4°C. below the rectal temperature, this relationship is reversed during the passage of high frequency currents of strengths equivalent to those usually employed in therapy. The lung temperature now exceeds the rectal temperature, but only by a few tenths of a degree. Moreover, there is a tendency for the lung to cool and gradually to approximate the rectal temperature again unless the current strength is augmented. This is well shown in Fig. 4, where the lung temperature crosses the rectal temperature first at the start of current flow, gradually falling below it, but again crosses it when the milliamperage is increased. In the light of subsequent experiments this gradual fall in lung temperature may be interpreted as resulting from increased blood flow through the lung and increased pulmonary ventilation.

The fact that death allows a sudden increase of heat in the deep tissue between the electrodes, and that this occurs in the abdominal organs as well as in the thoracic, suggests that the circulation rather than the respiration is chiefly responsible for the removal of heat from the lungs. Whether or not the heating of deep tissues is the result of the passage of current through them or is due to conduction from the more superficial structures has not yet been established. This question will be considered in another communication.

SUMMARY AND CONCLUSIONS.

1. Experimental evidence is furnished to show that in normal animals the rectal temperature can be elevated by the passage of high frequency currents.

2. During life the intraabdominal and intrathoracic temperatures can be increased only slightly above the rectal temperature.

3. The lung temperature in the anesthetized dog normally lies 0.3-0.4°C. below the rectal temperature. During the passage of diathermy currents of strengths equivalent to those used in therapy this relationship is reversed—the lung temperature exceeding the rectal temperature by about the same value.

4. Immediately after death, the temperature rises abruptly in the deep tissues between the electrodes.

5. For the measurement of deep temperature special thermocouples have been devised. Their method of preparation and mode of use are described.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

II. The Conditions Necessary for the Production of Local Heat in the Lungs.

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It has been shown elsewhere (1) that the passage of diathermy currents of strengths equivalent to those used in therapy raises the deep temperature in those portions of the abdominal and thoracic viscera which lie between the electrodes only slightly (about 0.4°C.) above the rectal temperature. That the rectal temperature can be elevated in both anesthetized and unanesthetized animals was also shown. In one unpublished experiment (D 27) the rectal temperature of an anesthetized dog as recorded by a thermocouple was raised from 37.71°C. to 40.48°C. during the passage of a current of 2000 milliamperes for 45 minutes between electrodes placed laterally on the thorax. The current density here was 166 milliamperes per square inch of electrode surface, which exceeds that commonly employed for therapeutic purposes, and yet at the end of the period the lung temperature in four different parts of the lungs, as recorded by four thermocouples, was 40.83°C., 40.85°C., 40.85°C., and 40.85°C., or 0.37°C. above the rectal temperature. A measurement of rectal temperature by a mercury thermometer made at the same time agreed exactly with the rectal thermocouple reading.

Furthermore, we have shown that sudden death permitted an abrupt rise of local temperature in the deep tissues between the electrodes without a corresponding rise in rectal temperature. This was observed in both intraabdominal and intrathoracic tissues, a fact which, together with the tendency of the lung temperature to approximate that of the rectum, suggested that the cooling of the lung was primarily a circulatory phenomenon rather than one due to the passage of air in and out of the lung.

To put this hypothesis to the test experiments were devised which had for their purpose a study of those conditions which would permit local heating of the lung during life.

EXPERIMENTAL.

In the part to follow experiments will be described in which the access of air or blood or both to one lung was prevented by various operative procedures. Temperature measurements were made in the altered lung with simultaneous records of temperature in the opposite lung, as well as in other parts of the body. It was discovered, as was anticipated, that the circulating blood, rather than the access of air, played the chief rôle in carrying away heat produced by diathermy.

Experiment D 14. Effect of Diathermy on Lung Temperature during (a) Blocking of the Left Bronchus, (b) Occluding the Left Branch of the Pulmonary Artery.— A male mongrel weighing 9.7 kilos was anesthetized by the intravenous injection of 2.91 gm. barbital-sodium dissolved in physiological saline. An additional 8 cc. of a 5 per cent solution of the drug was required to obtain complete anesthesia and relaxation. Tracheotomy was then performed. With the dog lying on his right side a thoracotomy was done through the fifth left interspace. A brass tube with flanged end was passed down the trachea and securely tied into the left bronchus just beyond the tracheal bifurcation. A similar, but shorter, brass tube with an equal bore, 7 mm., was inserted into the trachea parallel to the first one. A firm ligature was placed about the cut trachea so that both tubes were held firmly in place, and no air leaked about them.

By this procedure the air was partitioned, that passing down the one tube entering the left lung, that passing down the other entering the right lung. The access of air to either lung could therefore be prevented[1] by closing the mouth of the tube. To interrupt the blood flow to the lung a pneumatic cuff of the kind recently described by Moore and Binger (2) was placed about the left branch of the pulmonary artery and a tube communicating with the cuff brought out through the chest wall. By blowing air through this tube the cuff was inflated, thus effectively occluding the artery.

Lead-tin electrodes were applied laterally to the chest walls, care being taken to have the surfaces as parallel as possible. Several layers of gauze soaked in glycerol-salt mixture were interposed between the electrodes and the shaved skin to assure perfect contact. Thermocouple needles were then inserted into

[1] We wish to thank Dr. Richmond L. Moore, of this laboratory, for providing us with this technique.

the dog's lungs in the manner previously described (1). At the close of the experiment it was shown that two of the thermocouples lay in the left lower lobe, one in the right lower lobe, and one in contact with the right visceral pleura, probably in the interlobar space. A mercury thermometer was used to record rectal temperature.

After control readings of all temperatures had been recorded the current was turned on and its strength increased until the ammeter read 1200 milliamperes. After 40 minutes flow the rectal temperature rose by 1.35°C., the temperature in the right lung by 1.18°C., and the temperature in the left lung by 1.31°C. The access of air to the left lung was then prevented by blocking the appropriate tube. No change in rate of heating of the left lung resulted from this procedure. In the succeeding half-hour the rectal temperature rose by 1.21°C., the right lung by 0.95°C., and the left lung, whose bronchus was now blocked, by 1.02°C.

By opening the end of the tube the bronchial obstruction was released and air again permitted to enter the left lung. 11 minutes later the rubber cuff surrounding the left branch of the pulmonary artery was inflated, thereby shutting off part of the blood supply to the left lung. 5 minutes before interrupting the circulation in the left lung the dog's rectal temperature was 38.47°C., 5 minutes after, 38.88°C. The corresponding lung temperatures were—right, 38.63–38.85°C., left 38.73–40.80°C. In other words, whereas the temperature of the right lung rose 0.2°C. in this interval the temperature of the left lung suddenly rose 2.0°C. as the result of clamping the left branch of the pulmonary artery. The subsequent rate of heating of the two lungs was the same.

Later in the experiment the left bronchus was again occluded with no apparent effect on the rate of heating of the left lung. The animal was then suddenly killed by the intravenous injection of 20 cc. of chloroform. There resulted an abrupt and equal rise in the heat production in both lungs, the rectal temperature in the meanwhile falling. The temperature curves are shown in Fig. 1.

This experiment (D 14) is presented in some detail because it contains in it all the facts which are to be analysed in the remaining parts of this paper. The general conclusions suggested by it are these: (1) Prevention of access of air to one lung does not alter its rate of heating by diathermy, nor does it permit local heating in excess of the general systemic heating. (2) Interruption of the circulation through a branch of the pulmonary artery does, however, result in a sudden rise of temperature in the lung supplied by this branch. After the sudden inital rise heating continues at the original rate which in general is the rate of heating of the other lung and of the rectum. (3) Sudden death of the animal during diathermy results in a precipitous and equal increase in the temperature of both

lungs, the one with its artery occluded as well as the normal lung, and in a gradual fall in rectal temperature.

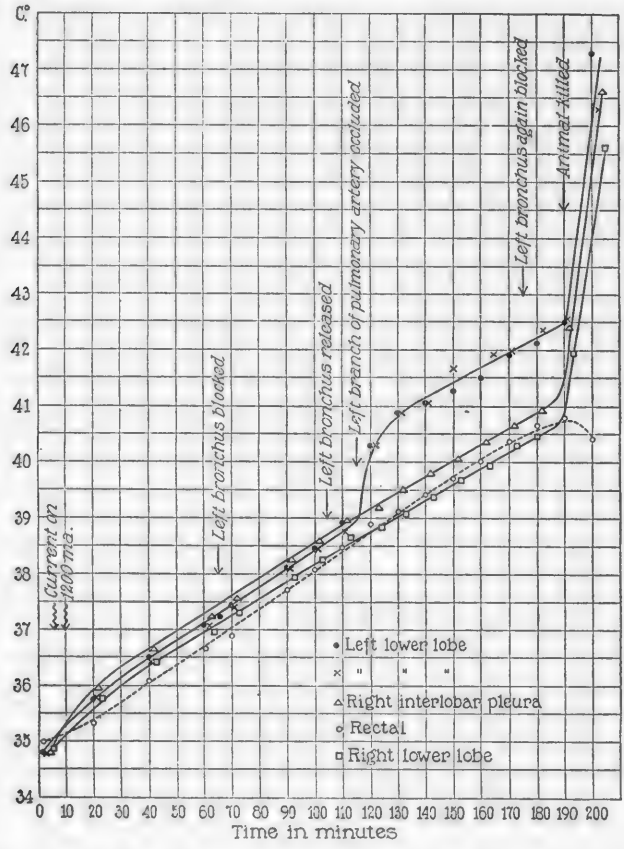

FIG. 1. Curve showing effect of (*a*) obstructing left bronchus, (*b*) occluding left branch of pulmonary artery, on heat developed in left lung as compared with heat developed in the right lung and rectum.

A further analysis of these various points follows:

Experiment D 21. Effect of Bronchus Blocking on Heat Production in the Lung. —A female collie weighing 9.7 kilos, anesthetized with barbital-sodium given

intravenously, was operated upon for the purpose of intubating the left bronchus. A diathermy current of 1200 milliamperes was passed between electrodes placed laterally on the chest. Temperature readings were made on thermocouples in the right and left lungs. During the current flow the left bronchus was blocked and subsequently released. The temperature in the right lung was 39.74°C., and in the left, before blocking the bronchus, was 39.75°C. 13 minutes after blocking the access of air to the left lung, it had warmed 0.94°C., the right lung 0.83°C. The maximum increase in temperature of the left lung over the right during the period of blocking was 0.38°C. Whether this is a real effect or due to experimental error, we do not know. It is probably a real one as the agreements in lung temperatures in this experiment, as well as in other ones, before blocking, were closer than this. In any case it shows, as does the last experiment, that prevention of access of air to one lung during diathermy of the thorax does not provide conditions which make for any significant degree of local heating.

Experiment D 15. Repetition of Arterial Occlusion Experiment.—It has already been shown that clamping the artery to one lung during diathermy results in a sudden increment of local heat. A second experiment on an anesthetized dog was performed with a pneumatic cuff placed about the left branch of the pulmonary artery. This cuff was alternately inflated and deflated with immediate changes in heat production in the lung.

The experimental results are so clearly shown in Fig. 2 that it seems superfluous to describe them at greater length. Four things should be noticed in particular: First, the smoothness of the curves; secondly, the fact that once the sudden rise has occurred there is a tendency for the lung to continue heating up at approximately the original rate; thirdly, that after clamping the artery to the left lung the temperature of the right lung falls below the rectal temperature to an extent not seen in experiments in which the pulmonary circulation is not disturbed. This is an interesting point which is also shown in Fig. 1. It probably signifies that on interrupting the circulation to the left lung there is an increased flow of blood through the right lung, with consequently more efficient cooling. The fourth point of interest shown in Fig. 2 has been mentioned before—it is the sudden heating up which occurs equally in the two lungs, upon the death of the animal.

An explanation for some of these facts is furnished by the next experiment, in which the root of one lung was ligated during diathermy. This was done for the purpose of interrupting the circulation through the bronchial artery, as well as through the pulmonary artery. It was

thought that in all probability the blood flow through the bronchial vessels would be sufficient to maintain some degree of cooling even

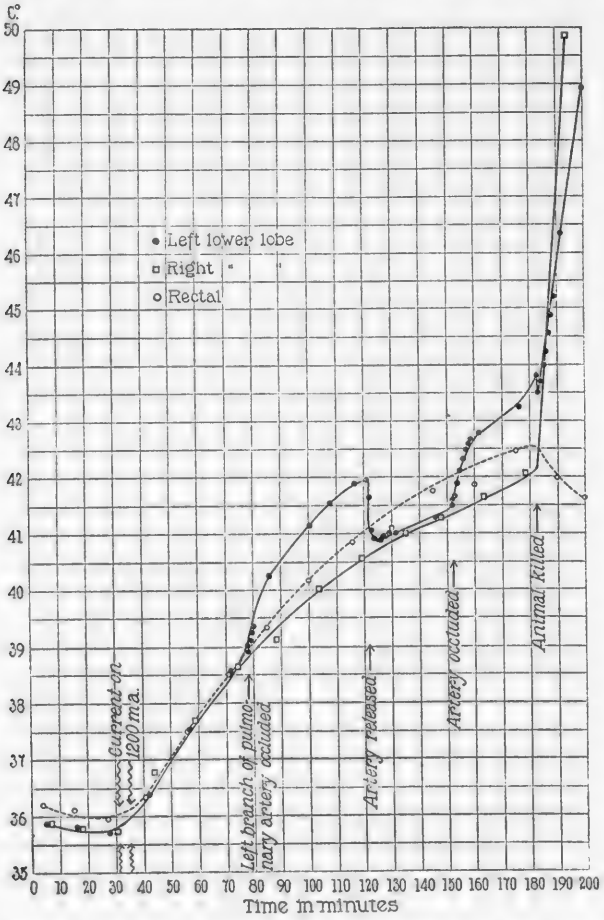

FIG. 2. Curve showing comparison of heat developed in right and left lungs and rectum, with effect of clamping and releasing artery to the left lung.

after the circulation through the pulmonary artery had been shut off, and thus prevent the temperature in the lung from rising precipitously as it does at death.

Experiment D 17. Ligation of Root of Right Lung during Diathermy.—A female fox-terrier under barbital anesthesia was prepared for the experiment by a right sided thoracotomy in which a loose ligature was placed around the root of the right lung. The ligature ends were brought out through the deep muscles of the back in such a manner that subsequent traction and ligation of the ends would completely shut off the circulation to the right lung. After repair of the chest wall and application of the electrodes, thermocouples were inserted into both lungs. At the close of the experiment the position of the thermocouples was confirmed. The animal was put on artificial respiration by the intratracheal insufflation of an interrupted stream of air. A current of 1200 milliamperes was then allowed to pass between the electrodes. In the half-hour previous to vessel ligation the right lung temperature rose from 36.12°C. to 40.04°C. These figures represent mean readings of two thermocouples in the right lower lobe. Simultaneously the temperature in the left lower lobe had risen from 36.21°C. to 40.32°C., showing that the two lungs were heating at approximately the same rate. The change in rectal temperature was of a similar grade.

The ligature about the right hilum was then suddenly tightened, with the result that in the next 6 minutes the right lung temperature increased by more than 2°C., the left by 0.6°C. 15 minutes later the right was over 3.5°C. warmer than the left. After killing the animal the rate of temperature increase in the right lung remained relatively constant, while the temperature in the opposite lung suddenly mounted precipitously. The changes are graphically shown in Fig. 3.

In another and similar experiment (D 19) the left lung root vessels of a dog were ligated before diathermy, leaving the bronchus, however, free. A current, again of 1200 milliamperes, was passed through the dog's thorax, and temperature measurements were made. In this experiment, as will be seen in Fig. 4, the right lung temperature increased at the same general rate as the rectal temperature; the left lung, on the other hand, at a much faster rate. Subsequent ligation of the left bronchus had little influence on the rate of warming of the left lung, whose root vessels had previously been tied. The dog was killed by the intravenous injection of 35 cc. of chloroform. This resulted in a precipitous rise in temperature in the unligated lung similar to that which had occurred in the ligated lung when the current was first turned on.

These last two experiments, we believe, indicate that the bronchial circulation may play an important part in carrying away the heat produced in the lung by the passage of high frequency currents. The blood flowing through the bronchial artery normally drains into the pulmonary veins. By ligating these structures at the lung root the whole circulation to the lung can be stopped, which cannot be accomplished by ligation of the main branch of the pulmonary artery alone. When the circulation to the lung is thus interrupted (even though its

bronchus remains patent) it is heated rapidly by the diathermy current to a point several degrees above the temperature prevailing in the

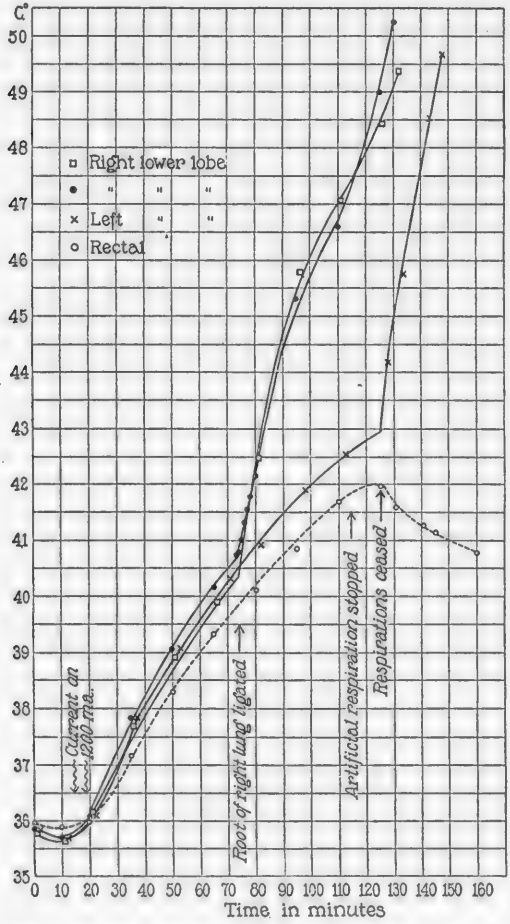

FIG. 3. Curve showing comparison of heat developed in right and left lungs and rectum. Effect of ligating the root of the right lung, and effect of sudden death.

normal lung or in the rectum, and at a rate equivalent to the rate of heating which occurs in the normal lung after death.

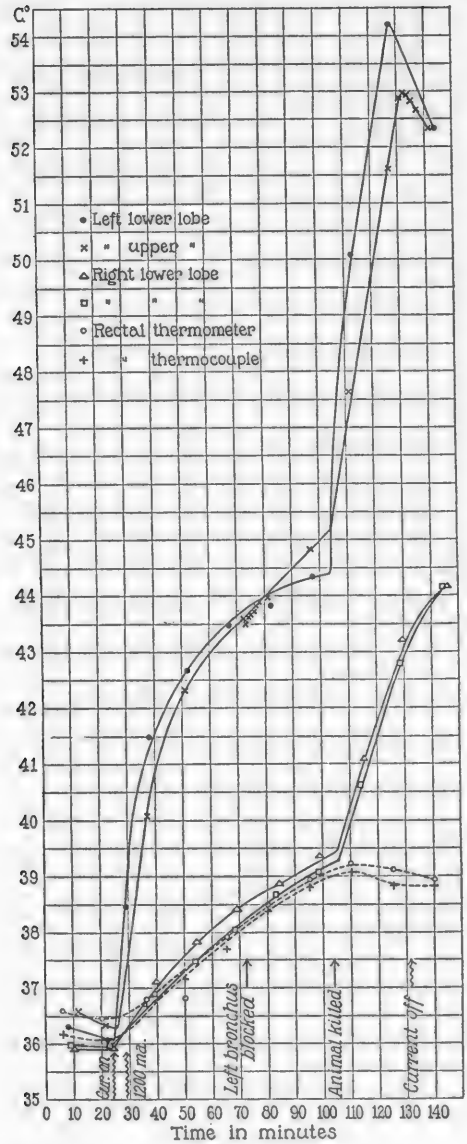

FIG. 4. Curve showing relative rates of heating of normal lung and lung whose root vessels have been ligated.

DISCUSSION.

Under the circumstances of these experiments, at least, it seems apparent that blood cooling is a more effective mechanism in removing heat from the lung than air cooling. Indeed, it is probable that the condition most needed for the production of local heat in the lung, by the passage of the diathermy current, is a partially or completely obstructed circulation in the pulmonary and bronchial vessels. The bearing of this on the problem of local heat production in the pneumonic lung is obvious, and will be experimentally dealt with later.

SUMMARY AND CONCLUSIONS.

1. Prevention of the access of air to one lung, while its circulation is intact, results in little, if any, change in the rate of heating of the lung by the diathermy current.

2. Occlusion of a main branch of the pulmonary artery during the flow of the current results in a sudden rise in temperature in the lung whose artery has been occluded, with subsequent heating, however, at the original rate. Under these circumstances death of the animal is accompanied by a precipitous rise in the temperature of both lungs.

3. When the pulmonary veins as well as the artery to one lung are ligated the circulation through the bronchial vessels is also stopped. Diathermy then results in a local rise in temperature in the lung equivalent to that seen in the other lung after death.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

III. The Temperature of the Circulating Blood.

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We have convinced ourselves by experiment that the normal lung can be heated only slightly above the systemic temperature by the application of high frequency currents to a dog's thorax (1). Interference with the circulation of blood to the lung, however, either by clamping one of the main branches of the pulmonary artery, or by ligation of all the root vessels, provides the conditions necessary for local deep heating (2). The implication that the circulating blood serves to carry away the heat produced in the lung seems obvious. Such an interpretation is in harmony with the physiology of heat distribution and temperature regulation (3). It is probable that together with its many other equilibrating functions the blood is a fairly ideal medium for distribution and maintenance of a uniform temperature.

Methods.

The methods which we have previously used for deep temperature measurements were easily modified for the purpose of recording intravascular temperatures. In place of the thermocouple needle we used similar copper-constantan couples housed in No. 14 gauge rolled steel tubing 10 cm. long. The junction was soldered to the end of the tubing, the solder forming a smooth, blunt tip. Capillary rubber tubing with a 3 mm. outside diameter was used to protect the wires. This was sufficiently small to permit its passage through the femoral vein or artery into the vena cava and aorta, or through the jugular and carotid, into the right and left heart. The steel and rubber tubing were smeared with a petroleum jelly of high melting point to inhibit blood clotting about them.

The methods for applying diathermy were those previously described.

EXPERIMENTAL.

The Temperature of Arterial and Venous Blood.

Experiment D 24.—A male mongrel hound weighing 21.7 kilos was anesthetized by the intravenous injection of barbital-sodium. The right femoral artery and left femoral vein were exposed and opened sufficiently to admit the passage of two thermocouples into their lumina. These thermocouples were then pushed up into aorta and vena cava. At the conclusion of the experiment the aortic thermocouple was found in the abdominal aorta just under the diaphragm; the other in the vena cava at the opening of the superior hepatic vein. The dog's rectal temperature was recorded by a mercury thermometer calibrated in 0.10°C., which could be read accurately to about 0.02°C. A comparison of rectal thermometer readings and temperatures in the right femoral artery and aorta showed an average agreement within 0.02°C. in five readings taken at approximately 5 minute intervals for ½ hour. This agreement held even though the thermocouple position was altered and advanced up the artery from a point 10 cm. above its entrance into the vessel to a point 30 cm. above its entrance into the vessel. The latter position was the one found at autopsy, namely, in the abdominal aorta immediately under the diaphragm. It should be stated that when the animal began to lose heat and the rectal temperature fell, the agreement was not so close as that just mentioned.

Measurements of venous blood temperatures made simultaneously showed a decided difference from both arterial and rectal temperatures. At the time of the first three observations the venous temperature was 0.25–0.33°C. below the arterial. With the further advance of the thermocouple upward into the vena cava this difference became less until a point was reached 30 cm. above the entrance of the thermocouple into the vein, which corresponded to the level of the hepatic veins, where the venous temperature was higher than both arterial and rectal temperatures by slightly more than 0.10°C.

In the chambers of the heart the normal relationship, in the anesthetized dog at least, is for the temperature in the left ventricle to be slightly below the temperature in the right auricle. This difference amounts to less than 0.2°C. and usually more than 0.05°C., and may be supposed to be due to heat loss in the blood as it traverses the lungs. The difference between temperature readings obtained by passing thermocouples down the right jugular vein and left carotid artery into the heart in three dogs is shown in Table I. As will be understood from what has just been stated, the venous temperature will depend not only on the location of the thermocouple but on the relative amount of blood from the abdominal viscera flowing into the right auricle.

In another experiment (D 40) in which great care was taken to prevent cooling of the animal, the rectal temperature rose by nearly 0.1°C. during the period of observation. In this dog the temperature of the blood was lower than that registered by a thermocouple in the rectum. The venous and arterial blood temperature, however, exhibited the relationships just described. As the venous thermocouple was advanced along the femoral vein and up into the vena cava the recorded temperature continued to mount until it exceeded the arterial blood temperature by 0.04°C. Both intravascular thermocouples were then occupying positions 47.5 cm. above the points of entrance in the femoral vessels, one of them lying in the vena cava, the other in the aorta at a level half-way between the dome of the diaphragm and the heart. These relative temperatures are

TABLE I.

Comparison of Temperatures Recorded by Passing Thermocouples down Right Jugular Vein and Left Carotid Artery.

Experiment No.	Reading on thermocouple passed down right jugular vein	Reading on thermocouple passed down left carotid artery	Difference
	°C.	°C.	°C.
D 25	36.37	36.29	−0.08
	36.35	36.33	−0.02
D 26	34.91	34.87	−0.04
	34.81	34.75	−0.06
	34.75	34.69	−0.06
D 29	37.07	36.88	−0.19
	37.06	36.87	−0.19
	37.11	36.93	−0.18

graphically shown in Fig. 1. The upward inclination of the temperature curve for the arterial thermocouple was the result of the gradual rise in body temperature as illustrated by the similar curve for the rectal temperature. It should not be confused with the striking rise in venous temperature which occurs as the thermocouple is advanced upward into the vena cava.

The Temperature of the Arterial and Venous Blood during Diathermy.

The passage of the diathermy current between electrodes placed laterally on the dog's thorax results not only in an elevation of rectal temperature and lung temperature, as has been shown, but in an equivalent rise in the temperature of the circulating blood. The

arterial blood in the left heart or aorta, normally slightly cooler than the venous blood, rises to a greater degree than does the venous. The usual relationship is, therefore, reversed. For example, in Experiment D 25 the temperature in the left ventricle before diathermy was 36.29°C., in the right auricle, 36.37°C. After the onset of current flow this relationship was reversed, the temperature in the left heart now exceeding that in the right by 0.14–0.28°C. The difference, though not great, persisted as long as the current flow continued and disappeared as soon as the current was shut off, the

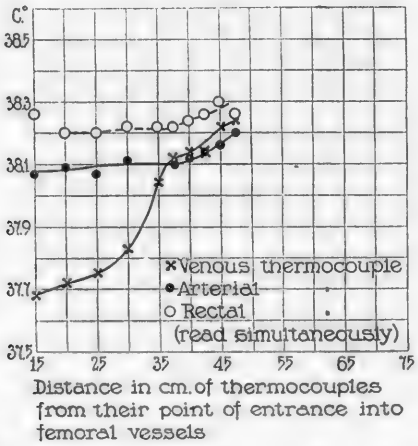

FIG. 1. Curve showing temperatures at various levels in aorta and vena cava with simultaneous rectal temperatures.

venous being now 0.07°C. above arterial. In another experiment (D 26) the left carotid blood was 0.06°C. cooler than the right jugular before the passage of 1500 milliamperes between the electrodes. After ½ hour's diathermy, the arterial blood temperature exceeded the venous by 0.2°C.

Changes of a similar order of magnitude appeared in Experiment D 40. These are graphically plotted in Fig. 2, and in Table II will be found a comparison of the temperatures of arterial and venous blood before and during the passage of the diathermy current in four separate experiments.

TABLE II.

Comparison of Arterial and Venous Blood Temperatures before and during Diathermy.

Experiment No.	Temperature before diathermy			Temperature during diathermy		
	Arterial	Venous	Difference	Arterial	Venous	Difference
	°C.	°C.	°C.	°C.	°C.	°C.
D 25	36.29	36.37	−0.08	39.70	39.47	+0.23
D 26	34.69	34.75	−0.06	41.83	41.73	+0.10
D 29	36.88	37.06	−0.18	38.95	38.99	+0.04
D 40	38.22	38.29	−0.07	40.01	39.89	+0.12

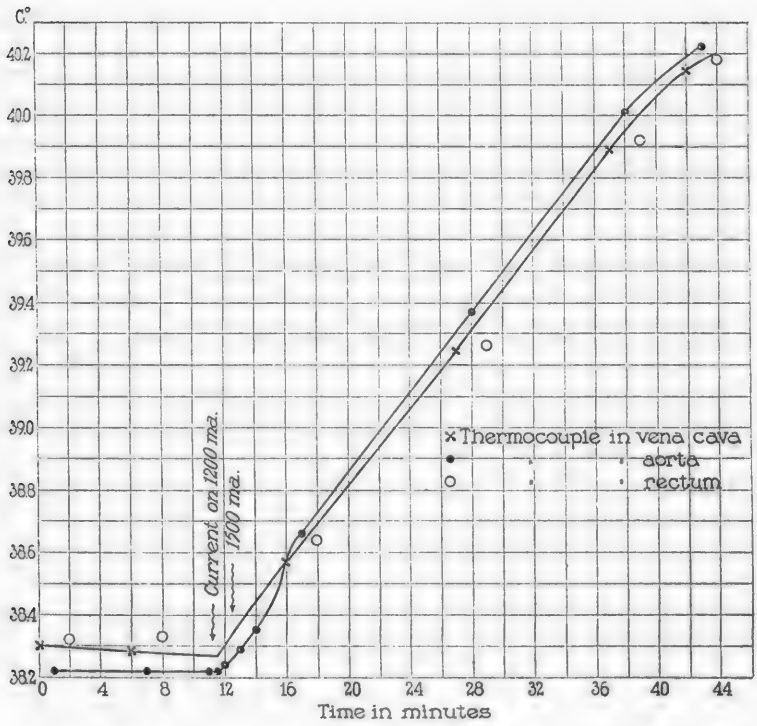

FIG. 2. Curve showing temperature of right and left heart blood before and during diathermy.

DISCUSSION.

The facts presented in this paper may be regarded as further evidence of the effective cooling of the lung during diathermy by the blood circulating through it. It can be estimated roughly, assuming a minute volume of blood flow through the lungs of 2.50 liters, and a rise of 0.2°C. in arterial blood temperature above venous, that approximately half of a large calorie of heat is being removed from the lungs per minute. This is evidently sufficient to prevent any marked degree of local heating.

SUMMARY AND CONCLUSION.

1. A method of measuring intravascular temperatures in anesthetized dogs has been described.

2. The temperature in the abdominal aorta is uniform throughout, and varies only with the systemic temperature.

3. The temperature in the inferior vena cava rises as the thermocouple approaches the heart, reaching its maximum at about the level of the hepatic veins. Between the hepatic veins and the right chambers of the heart there is no further elevation in venous temperature.

4. The temperature of the right heart blood normally exceeds that of the left heart blood by 0.05–0.2°C.

5. During the application of high frequency currents to the thorax, this relationship is reversed.

6. This indicates that the lungs are being heated but that the blood passing through the pulmonary vessels is removing the heat at approximately the rate of production.

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THE SOLUBLE SPECIFIC SUBSTANCE OF FRIEDLÄNDER'S BACILLUS.

III. On the Isolation and Properties of the Specific Carbohydrates from Types A and C Friedländer Bacillus.

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The parallelism between capsular synthesis and virulence among pathogenic bacteria is one which has been extensively investigated, but researches concerning the chemical nature of encapsulating substances have indeed been very few. Fürst (1) has described the encapsulating substance of the Friedländer bacillus. He believed it to be a mucoprotein-like substance. Hamm (2) contended that the capsulate substance contained nucleoprotein. Preisz (3) has attempted unsuccessfully to immunize animals with the capsular substance from the anthrax bacillus. More recently Toenniessen (4) succeeded in isolating a nitrogen-free polysaccharide from the Friedländer bacillus, which he believed to be identical with the capsular material of this microorganism. Similar polysaccharides have been isolated from cultures of the bacilli of the *aerogenes* group (5), from *Streptococcus hornensis* (6), and even from certain yeasts (7).

Strange as it may seem, none of these earlier investigators ascribed immunological significance to these bacterial carbohydrates. The so called soluble specific substance of the pneumococcus, first observed by Dochez and Avery (8), has been identified with the polysaccharide portion of the organism. An extensive investigation into the chemical and immunological nature of carbohydrates from the pneumococcus group has been carried out in this laboratory (9). Similar investigations have recently been extended to the group of Friedländer bacilli.

From a strain of Friedländer's bacillus, Mueller, Smith, and Litarczek (10) have recorded the isolation of carbohydrate-containing material which at high dilutions reacted specifically with homologous antibacterial serum. It has been shown by Julianelle (11)

that bacilli of this group are separable immunologically into sharply defined specific types. These types have been designated as Type A, Type B, and Type C; the remaining unclassified strains were placed in a heterologous group X. The method of isolation of the specific carbohydrate of Type B Friedländer bacillus has been given in detail (12). The present communication, which concludes a systematic description of the specific polysaccharides from the three fixed types of Friedländer's bacillus deals with a description of the polysaccharides from the Type A and Type C microorganisms. A more detailed study concerning the nature of the hydrolytic products of the Type A specific carbohydrate is reported separately (13).

EXPERIMENTAL.

I.

1. Isolation of the Specific Carbohydrate of Type A Friedländer Bacillus.

Autoclaved washings from 50 large Blake bottles of 72 hour cultures of Type A Friedländer bacillus, grown on solid agar containing 0.5 per cent dextrose at pH 7.6, were treated with a sterile solution of purified trypsin.[1] After standing overnight at 37° the mucoid-like washings (containing the specific carbohydrate) became nearly clear. The faintly alkaline solution was treated with 50 gm. of sodium acetate and 2 volumes of alcohol. A flocculent precipitate, containing all the specific carbohydrate in an impure state, settled out on standing. The supernatant liquid which gave no test for specific substance with antiserum, was discarded and the precipitate was separated by centrifugation. After dissolving the precipitate in a liter of water an opalescent solution was secured. By adding acetic acid it was found that a small amount of non-specific nitrogenous material could be separated by centrifugation. The solution of impure specific carbohydrate thus obtained was again treated with 2 volumes of alcohol and the resulting precipitate of specific carbohydrate was again separated by centrifugation. This process of alcoholic precipitation (a process which was found to eliminate nitrogenous impurities) was repeated five or six times until finally a snow-white product was secured, which gave a perfectly clear solution when dissolved in water. This solution, now at a volume of 500 cc., was treated with 50 cc. of 1:1 hydrochloric acid and was dialysed in collodion bags against successive changes of distilled water

[1] 8 gm. of commercial trypsin were dissolved in 200 cc. of water, acidified with N/1 hydrochloric acid until Congo red paper turned a faint purple. The solution was centrifuged, and the supernatant liquid was neutralized with N/1 sodium hydroxide, and again centrifuged.

until free from Cl ion both within and without the bag. The dialysed solution of the carbohydrate was concentrated to 200 cc. *in vacuo*, and was then treated with 400 cc. of redistilled glacial acetic acid. After standing 2 or 3 hours a flocculent precipitate settled out of the solution. This non-specific precipitate was separated by centrifugation and was discarded. The clear supernatant liquid, which contained all the specific material, was concentrated to small volume *in vacuo*, and was then poured into 10 volumes of redistilled acetone containing a trace of hydrochloric acid. Between 5 and 6 gm. of polysaccharide were recovered.

2. Properties of the Soluble Specific Substance of Type A Friedländer Bacillus.

The soluble specific substance of the strain of Type A Friedländer's bacillus is a white amorphous powder with marked acidic properties,

TABLE I.

Soluble Specific Substance of the Friedländer Bacillus Type A.

Preparation No.	$[\alpha]_D$	Acid equivalent	Ash	C	H	N	Reducing sugars on hydrolysis (as glucose)	Highest dilution giving precipitate with immune rabbit serum
							per cent	
117-A	−103.4°	430	0.0	43.98	6.00	0.1	64	1:2,000,000
118	−101°	430	0.0			0.0	65	1:2,000,000
120	−105°	445	0.0			0.0	68	1:2,000,000
122	−100°	435	0.0			0.1	65	1,2:000,000
123	−101°	430	0.0			0.0	67	1,2:000:000

the acid equivalent being approximately 430. A 1:200 solution turns Congo red paper blue. Such solutions are not precipitated by copper or silver ions. The polysaccharide is incompletely precipitated by solutions of phosphotungstic acid, barium hydroxide, and uranyl nitrate, but is completely precipitated by both neutral and basic lead acetate solutions. The nitrogen-free product, when oxidized with sodium peroxide, gives no test for sulfur or phosphorus. The polysaccharide gives no color test with potassium iodide-iodine solution. Acid hydrolysis of the material gives a reducing solution which shows a strong naphthoresorcinol test. Attempts were made to purify the specific substance by partial precipitation with barium hydroxide, uranyl nitrate, and by adsorption on alumina, but in each

instance a substance with properties similar to the original material was recovered. The properties of the various preparations were remarkably uniform as shown by Table I.

II.

1. Isolation of the Type C Soluble Specific Substance of Friedländer's Bacillus.

The specific carbohydrate of the strain of Type C Friedländer's bacillus was obtained from autoclaved washings of cultures grown on dextrose agar media exactly as was the carbohydrate from Type A. The product was isolated in an ash-free form by the addition of 2 volumes of alcohol to a 1:50 solution of carbohydrate, containing 10 cc. of 1:1 hydrochloric acid. After standing at 0° for 1 hour the polysaccharide separated as a flocculent precipitate which was filtered on a hardened paper and washed free from chloride with absolute alcohol. The yield from 100 Blake bottles was about 3 gm.

2. Properties of the Soluble Specific Substance of Type C Friedländer Bacillus.

The material thus isolated was an amorphous water-soluble powder, free from nitrogenous impurities and ash. The substance is a strong acid, for it turns moist Congo red paper blue. A 1:200 solution is not precipitated by solutions of silver nitrate, copper sulfate, phosphotungstic acid, or ammonium molybdate, but is precipitated by solutions of uranyl nitrate, neutral and basic lead acetates, and by concentrated solutions of barium hydroxide. The carbohydrate gives no color with iodine-potassium iodide solution. The substance has a specific rotation of +100° and its acid equivalent is approximately 680. It reacts with immune rabbit serum at a dilution of 1:2 million.

The properties of various preparations are shown in Table II.

3. Hydrolysis of the Type C Specific Substance.

1.5 gm. of Preparation 3-A were boiled for 5 hours with 50 cc. of N/1 sulfuric acid under a reflux. At the end of this time the sulfuric acid was quantitatively removed with barium hydroxide, the solution was boiled with an excess of calcium carbonate and a small amount of norit, and was filtered. The clear filtrate was evaporated to dryness *in vacuo*, and the residue was extracted with methyl alcohol, the alcoholic solution was filtered and was then evaporated to dryness *in vacuo*. (In this manner the free sugars were separated from the sugar acids, which were

present as their calcium salts and which were insoluble in methyl alcohol.) The residue of free sugars was dissolved in water and diluted to 100 cc. in a volumetric flask. An analysis by the Shaffer-Hartmann method showed 0.84 gm. of reducing sugars calculated as glucose. On the basis of this weight the solution showed a specific optical rotation of +48.2°.

TABLE II.

Soluble Specific Substance of the Friedländer Bacillus Type C.

Preparation No.	$[\alpha]_D$	Acid equivalent	Ash	N	Reducing sugars on hydrolysis (as glucose)	Highest dilution giving a precipitate with immune rabbit serum
					per cent	
1	+90.0°	610	0.0	0.5	75.0	1:2,000,000
1-A	+100.0°	680	0.0	0.0	73.1	1:2,000,000
2	+100.0°	681	0.0	0.0	—	1:2,000,000
3	+85.0°	575	0.0	0.0	—	1:2,000,000
3-A	+101.0°	674	0.0	0.0	75.0	1,2:000,000
3-B	+58.0°	794	0.0	0.3	55.0	1:1,000,000

Preparation 1 was the first preparation isolated and had a rather high nitrogen content. The nitrogen was removed (Preparation 1-A) by precipitating the substance in a 1:100 solution with barium hydroxide saturated at 60° and then recovering the polysaccharide from the precipitate, by treating it with an excess of sulfuric acid, centrifuging off the barium sulfate, and reprecipitating the polysaccharide, in the presence of acid, with alcohol.

Preparation 3 was obviously impure. It was separated into two fractions by treating a 1·100 solution of the carbohydrate with a slight excess of barium hydroxide saturated at 50°. The precipitate was centrifuged off and the carbohydrate was recovered in the usual way (Preparation 3-A). The supernatant liquid from this barium hydroxide precipitation still contained specific material. This was removed by precipitation with alcohol. The precipitate, Preparation 3-B, was obviously an impure substance still containing specific material. Attempts at further purification of the preparations by adsorption on alumina, precipitation with uranyl nitrate, etc., yielded products with properties identical with the starting material.

Half of the solution was treated with 3.5 mols of phenylhydrazine acetate and an osazone was isolated in the usual manner. 0.1 gm. was recovered. The substance melted at 201–203° and had an $[\alpha]_D = -54.3°$ mutarotating to −20.0° after 48 hours.

The second half of the solution was evaporated to 1 cc. and was oxidized with

3 cc. of 1:1 nitric acid. 0.070 gm. of potassium acid saccharate was obtained which was recrystallized from 1 cc. of water. 0.030 gm. was recovered.

0.005326 gm. substance gave 0.001853 gm. K_2SO_4.

Calculated for COOH $(CHOH)_4COOK$, K 15.75 per cent.

Found K 15.58 per cent.

It is evident that this sugar, which represents the greater part of the hydrolytic products of this soluble specific substance, is glucose. 0.5 gm. of residue was left from the methyl alcoholic extract of the original total hydrolysate. This material appeared to be the calcium salt of a sugar acid. It was an impure substance, judging from its color. It gave a strong naphthoresorcinol test and a strong reduction test. It is possibly the salt of an aldobionic acid such as has been found among the hydrolysis products of other specifically reacting polysaccharides. The substance will be investigated further when more material is available.

DISCUSSION.

It is clear from the foregoing experiments that strains of Friedländer's bacillus of Types A and C yield, on fractionation, two chemically distinct nitrogen-free polysaccharides with highly specific properties. Both are strong acids and both contain glucuronic acid, or an isomer, within their molecules as shown by the naphthoresorcinol test. The polysaccharides themselves are non-reducing, but on hydrolysis with mineral acids they yield reducing sugars. In both instances, as in the case of the pneumococci, specific function and carbohydrate are apparently inseparable.

On comparing the specific carbohydrates from Type B and Type C Friedländer bacillus, an unusual similarity in properties is to be observed. These substances, however, possess two distinct differences. Immunologically they show no cross-relationship. Their solubility in water in the pure state and their whole behavior during purification are entirely different. In pure form the Type B substance is difficultly soluble in water whereas the Type C substance is readily soluble. The Type B carbohydrate may be readily precipitated by alcohol in the presence of hydrochloric acid. The Type C substance, on the other hand, precipitates completely only after standing at 0° for an hour or more. The fact that two substances

so alike in physical properties are totally dissimilar in immunological reactions, may possibly be explained on the basis of slight differences in the intramolecular linkages of sugar to sugar, or of sugar to sugar acid.

SUMMARY.

1. Methods are given for the isolation of specifically reacting nitrogen-free polysaccharides from Type A and Type C Friedländer's bacillus.

2. The properties of these specific carbohydrates have been outlined.

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THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY.

IV. The Action of Diphtheria Toxin in Splenectomized and Blocked Mice.

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While the relation of the reticulo-endothelial system to the production of an artificially acquired immunity has been studied in the past from various angles, it is only recently that attempts have been made to bring phenomena of natural immunity into connection with the same system of cells. Our knowledge of the mechanism of natural resistance in certain animals to certain infections and intoxications has so far been limited almost entirely to the observation that such insusceptibility, with a few exceptions, is not due to humoral immune bodies and hence is not transmissible in most cases. In some instances, well defined physiological properties, such as a higher or a lower body temperature, can be held responsible for this phenomenon. Ehrlich did not actually advance our knowledge on this subject by attempting to explain the phenomenon on the basis of lack of suitable receptors in the tissue of the respective animals. In 1914, Lewis and Margot (1) published experiments which seemed to show that removal of the spleen from albino mice greatly increased their resistance to infection with *B. tuberculosis*. Later, Murphy and Ellis (2), however, were able to explain the anomalous results of the above mentioned authors. They found that mice splenectomized a short time prior to inoculation with *B. tuberculosis* were distinctly more susceptible than normal animals, while, with the increase of the interval between splenectomy and infection, the mice gradually showed a greater resistance than the controls. This increased resistance they explained by a secondary

hypertrophy of the remaining lymphoid tissue. Kyes (3) first definitely connected natural resistance with the fixed phagocytic cells of the endothelial system by his observations on the rôle of the hemophages of the liver and spleen in the natural immunity of the pigeon to pneumococcus infection. In 1925 Singer (4) reported on a breach of natural immunity effected by blockade of the reticulo-endothelial system. He was able, after blocking the reticulo-endothelial system of hens by intravenous injections of India ink, successfully to infect the fowls with *B. anthracis*, while the non-blocked controls remained refractory. Jelin's (5) recent publications likewise emphasize the importance of the reticulo-endothelial system for the mechanism of natural immunity against saprophytic organisms and more particularly for the conditions which determine the course of typhoid fever infection and intoxication in rabbits.

The relative insusceptibility of rats and mice to diphtheria toxin is well known. In the case of mice, it was first observed by Loeffler (6) in 1884 and has since been confirmed by a great number of authors. Roux and Yersin (7) and Behring and Kitashima (8) have been able to kill mice only by employing extraordinarily large doses of diphtheria toxin, while Loeffler failed entirely to infect them. Glenny and Allen (9) found that, when administered intravenously, the M.F.D. of a particular diphtheria toxin for mice was 60 times the subcutaneous M.F.D. for the guinea pig, while 100 times this dose had to be given to kill mice by the intramuscular route. Kolle and Schlossberger (10) reinvestigated the question and concluded that mice were practically insusceptible to large doses of diphtheria toxin, but were generally susceptible to fairly small doses of living bacilli. These authors also found no antitoxin in the blood of mice as had previously been described for rats by a large number of authors (Aronson (11), Koudrevetzky (12), Kuprianow (13), Cobbett (14), Goodman (15), Petitt (16), Coca, Russel, and Baughman (17)). Hippke (18) has been able only partially to confirm the one contention of Kolle and Schlossberger since he found but six strains, out of fifteen, to be virulent for mice. Members of the British Medical Research Council (19) also repeated Kolle and Schlossberger's work, using the same and even larger doses of diphtheria bacilli without obtaining regular results. Wolff (20), on the other hand, corroborates Kolle and Schlossberger's statements regarding the susceptibility of mice to the infection with living bacilli, and furthermore reports that he has succeeded in killing mice by repeated intravenous or intramuscular injections of fairly large doses of diphtheria toxin.

From a review of literature it would appear that the mouse is, to a large extent, insusceptible to diphtheria infection and intoxication,

although this immunity is by no means complete. It has seemed of interest to investigate whether the natural immunity of the mouse to diphtheria toxin and diphtheria bacilli is dependent upon the presence of an intact reticulo-endothelial system, by comparing the action of a highly potent diphtheria toxin and of a highly virulent diphtheria strain in normal mice with their action in mice which have been (1) blocked by intravenous injections of India ink, (2) splenectomized,[1] (3) blocked and splenectomized.

EXPERIMENTAL WORK.

Diphtheria toxin with and without phenol was used as well as diphtheria toxin concentrated by ultrafiltration. For the preparation of the latter we are indebted to Mr. D. Roelkey of this laboratory. The toxin was given by the intraperitoneal and the intravenous route in different experiments. The mice ranged in weight between 24 and 30 gm. Inasmuch as the pathological lesions at autopsy were not as characteristic as seen in other species, cultures were made on a blood plate from the heart's blood and the peritoneal exudate of the dead animals in order to disclose any intercurrent infection.

Series I.—Phenolized diphtheria toxin, No. 411a, having a M.F.D. of 0.0025 cc. for the guinea pig, was employed in this series. It was given intraperitoneally in amounts of 400 M.F.D. (1 cc.), 300 M.F.D. (0.75 cc.), 200 M.F.D. (0.5 cc.), and 100 M.F.D. (0.25 cc.), the volume of the dose in each case being 1 cc. Four groups of mice received these doses of toxin, each group consisting of eight animals: two normal controls, two animals which had received an intravenous injection of India ink (1 cc. 1:15) 2 days previously, two animals which had been splenectomized the day before, and two animals in which the ink injection had been combined with splenectomy. The eight mice which were injected with the 400 M.F.D. died in from 25 to 51 hours, those injected with 300 M.F.D. were found dead after from 34 hours to about 10 days. It should be stated, however, that in the latter group culture from the animal dying after 34 hours revealed the presence of hemolytic streptococci in both the heart's blood and the peritoneal exudate. In the group which received 200 M.F.D. death occurred in from 8 to 17 days, while all the mice which had received 100 M.F.D. survived. There was no definite and uniform difference between the control and the experimental animals in the length of time they lived after injection of the various doses of toxin. Death occurred rather irregularly within each group, demonstrating the wide margin of individual varia-

[1] The operations were performed by using ether as an anesthetic.

tion in susceptibility of mice to diphtheria toxin as compared with the uniform reaction of guinea pigs to this poison.

Series II.—Diphtheria toxin without phenol was used in this series in which the effect of intravenous injections was studied. Toxin without preservative was selected, because it was found in preliminary experiments that phenolized toxin (0.5 per cent) killed mice immediately in intravenous doses of 0.75 cc., and even doses of 0.5 cc. provoked an extremely violent, immediate reaction. Broth, without phenol, injected in similar amounts intravenously, caused only a very slight transitory peptone shock. This toxin, which had a M.F.D. of 0.002 cc. for the guinea pig, was given in a uniform volume of 1 cc. in amounts of 200 M.F.D. (0.4 cc.), 150 M.F.D. (0.3 cc.), 100 M.F.D. (0.2 cc.), and 75 M.F.D. (0.15 cc.) intravenously to four similar groups of mice arranged as in the first series. The eight mice which had received the 200 M.F.D. died in from 4½ to 7½ days, while death occurred in the second group which had received 150 M.F.D., in from approximately 5 to 9 days. With the mice injected with 100 M.F.D. death was prolonged until about 11 days, while in the last group which had received 75 M.F.D., only one normal mouse died after 9½ days. Again, death occurred within each group rather irregularly among duplicate animals, and no significant difference between experimental and control animals became apparent in the length of time they lived.

Series III.—Finally, a last series of experiments was carried out with diphtheria toxin (411a) which had been concentrated by ultrafiltration from an original volume of from 60 cc. to 6 gm. The original M.F.D., for guinea pigs, of 0.0025 cc. was thus decreased, as determined by animal tests, to 0.00025 cc. By using this concentrated and refined product it was possible to inject larger amounts intravenously without fear of a reaction induced by the excess of protein and phenol. Doses of 600 M.F.D. (0.15 cc.), 400 M.F.D. (0.1 cc.), 200 M.F.D. (0.05 cc.), and 100 M.F.D. (0.025 cc.) were injected intravenously in a uniform volume of 0.5 cc. into thirty-two mice, arranged in four similar groups as described before. With the first group which had received 600 M.F.D., death occurred in from 20 to 88 hours, in the second group (400 M.F.D.), the animals lived from 29 hours to 6 days. 200 M.F.D. caused death in from 5½ to 8½ days, while in the last group (100 M.F.D.) one splenectomized and one normal mouse survived; the others died in from 8½ to 13½ days. This series was likewise marked by the absence of any consistent difference in the time of survival between controls and experimental animals.

A small series of experiments (six animals) in which the course of infection with a highly virulent diphtheria strain[2] was studied in normal mice and in mice which had been either injected with India ink or splenectomized, disclosed no difference of susceptibility, between the normal and experimental animals. It was found that

[2] This strain killed guinea pigs weighing from 230 to 280 gm. in a dose of 1/20 slant within from 2 to 3 days.

doses as large as a whole slant of a 24 hour culture on Loeffler's medium were ineffective in producing in either group of animals any symptoms more marked than a small nodule at the site of injection.

DISCUSSION.

The results of the experiments described indicate that such elimination of the reticulo-endothelial system in the mouse as is accomplished by means of one blocking injection of India ink, or splenectomy, or a combination of both operations, has no influence on the natural resistance which this species possesses against diphtheria intoxication and infection. The conclusion which suggests itself is that in this case the mechanism of natural immunity differs fundamentally from the processes of artificially acquired immunity.

SUMMARY AND CONCLUSIONS.

1. The minimum amount of diphtheria toxin which killed normal mice of from 24 to 30 gm. in weight upon intravenous injection, was found to be between 75 and 100 times the M.F.D. for the guinea pig. When given intraperitoneally, the fatal dose for mice was as high as 200 M.F.D.

2. There was no significant difference in the lethal action of diphtheria toxin for normal mice and mice in which an elimination of the reticulo-endothelial system had been attempted by means of blocking injections of India ink, or splenectomy, or a combination of both operations.

3. Attempts to infect normal mice and mice treated as described with large doses of a highly virulent diphtheria strain were unsuccessful with both groups of animals.

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A STUDY OF THE PERIVASCULAR TISSUES OF THE CENTRAL NERVOUS SYSTEM, WITH THE SUPRAVITAL TECHNIQUE.

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PLATE 24.

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It has long been the accepted view that the ectodermal elements of the nervous system are separated by a compact membrane from the mesodermal tissues which accompany the blood vessels into the mass of the brain. Nissl (p. 345) held that this membrane, the membrana limitans gliæ, can be penetrated by mesenchymal cells only after it is damaged by the action of a severe pathological process.

Recently, however, del Rio-Hortega has questioned this view. Using the so called "silver carbonate" methods, which he has developed, he has found that a special manipulation of the technique brings out certain small cells which he terms the "microglia." In the adult, he finds these cells mingled everywhere with the ectodermal tissues of the brain. In the new-born animal, they seem to be concentrated in certain vascular zones of invasion, and from these regions he traces their wanderings into the ectodermal parenchyma. He brings evidence to show that these cells are the phagocytes of the nervous system, that they are identical with the phagocytes of the whole body, and that despite the fact that they seem to mingle freely with the ectodermal elements of the adult brain, they are none the less mesodermal in origin. Many workers, however, are still unconvinced as to the mesodermal nature of these cells. For this reason it has seemed desirable to apply to the central nervous system the supravital technique with which the phagocytic cells of the blood and of the general connective tissues of the body have recently been studied so minutely.

Vital methods have been used in the study of the phagocytes of the

central nervous system ever since the work of Bouffard, and of Goldmann. MacCurdy and Evans, and Macklin and Macklin used trypan blue, injected intravenously or intraperitoneally, to reveal the presence of dye-filled phagocytes in the brains of monkeys suffering from poliomyelitis and of rats subjected to sterile puncture wounds. The studies demonstrated that the phagocytes around brain lesions can take up particulate matter which reaches them through the vascular system. The nature and origin of the cells themselves are not settled by this observation and can be more directly determined by supravital methods.

EXPERIMENTAL.

The supravital technique has been applied to a series of sixteen normal animals, of which five were new-born rabbits, five guinea pigs under 7 days old, and six adult guinea pigs. Sterile puncture wounds were produced with a hot needle in four young anesthetized adult rabbits and in two guinea pigs; and an herpetic encephalitis was set up with herpetic virus of the strain "H.F." (described by Flexner and Amoss), by corneal inoculation in seventeen guinea pigs and twelve rabbits. After the supravital examinations were completed, all brains were fixed for subsequent study of stained sections. In this way was sought the ruling out of spontaneous disease in the "normal" animals while the extent of the lesions in the pathological material was established.

The supravital technique consists in the examination of the reaction of living cells to vital dyes immediately after removal of the cells from the body. Vital dyes, *i.e.*, neutral red and Janus green, were supplied to the cells in two ways: (*a*) In most of the experiments, fine films of dry neutral red and Janus green were prepared on slides according to the method described by Sabin, and by Sabin and Doan. When a minute amount of living tissue is placed on such a slide, the dye goes into solution in the tissue juices and reacts with the cells. (*b*) In six experiments, 300 to 500 cc. of dye (diluted 1:10,000 in physiological saline) were injected slowly through the aorta, after opening the cranium and the dura under anesthesia. In this way the brain could expand and become saturated with the dye, making it as certain as possible that the dye would reach every cell which had the power to react with it.

In obtaining the fragments of brain tissue, a standard technique was employed: (1) The brain was exposed under ether anesthesia. (2) The great vessels of the neck were opened to allow the brain to exsanguinate as completely as possible, so that the bits of brain tissue which were to be examined would be practically free from cells of the blood streams. (3) The dura and the pia-arachnoid were stripped away.[1] And (4) bits of brain tissue, about the size of a pin-head, were lifted out either with fine curved scissors, a sharp knife, or a capillary pipette. The bit of tissue was lifted out with as little damage as possible, without grasping with forceps, and was transferred gently to the slide which carried the film of dye. Under oil immersion magnification, the study of the preparation was undertaken at once in a warm box at body temperature. (5) In the meantime, the rest of the brain and some of the spinal cord were placed in a moist chamber in the warm box; and from time to time additional preparations were made, until an examination of as many regions as possible had been made. It was found that the brain would show little change under these conditions for nearly 2 hours, but that after this the new preparations would exhibit granulation, diffuse cellular and nuclear staining, and globules of free myelin. Before this point was reached, therefore, the brain was placed in a fixative.

DESCRIPTION OF OBSERVATIONS.

If a supravital preparation is made of a body fluid, it will spread itself under the cover-slip by capillarity. In this way blood (Sabin; Sabin, Cunningham, Doan, and Kindwall) lymph (Kindwall), cerebrospinal fluid (Kubie and Shults), peritoneal fluid (Sabin, Doan, and Cunningham, 1924), lymph gland, spleen, and bone marrow punctures (Cunningham, Sabin, and Doan, 1924, 1925; Doan; Sabin, Doan, and Cunningham, 1925) have been studied. Material taken from the brain, however, is too solid to spread without help, so that very gentle pressure must be applied, flattening the tissue out slowly and with a minimum of disruption. A preparation of this kind is always uneven. Some areas remain too dense for analysis; while in others the cells become entirely separated from one another. Of these separated cells, many remain morphologically undamaged, and are then particularly easy to study; but if injury occurs, it is readily recognized by the marked granulation of the cytoplasm and the rapid diffuse staining of the nucleus. (This usually happens around the edges of every preparation.) Many areas remain, however, which are neither too thick

[1] This is essential since, in a previous study (Kubie and Shults), these membranes have been found to contain phagocytes normally.

nor too thin, but in which the relations of various structures are well preserved, while the material is nevertheless thin enough for accurate observation under the highest oil immersion magnifications. And it is in portions of the preparations which have maintained their integrity in this way that the present observations have been made.

In these favorable areas, one special advantage of the supravital spreads is that the vessels and their surrounding tissues can be studied unshrunken and in their three-dimensional entirety, instead of in the single plane of a thin section of fixed material.

In the brains of normal animals, the perivascular meshwork contains at least two strains of cells which are readily recognized in the supravital preparations, the lymphocyte and the clasmatocyte. Lymphocytes are recognizable in three sizes: small, intermediate, and large. They take up little neutral red, and that in small vacuoles around the periphery. They have characteristic mitochondria, which vary from long, rod-shaped structures in the larger cells, which are probably the younger forms, to round dots in the smaller cells. They have a type of motility which is peculiar to themselves, in that they push their nuclei ahead of them, with a zone of thinner cytoplasm around the nucleus and the rest of the cytoplasm tailing out behind. This motility is observed chiefly in the intermediate size group.[2]

In contrast to the lymphocyte the mitochondria of the phagocyte are relatively obscure; while the vacuoles are abundant and prominent, and fill with neutral red in the presence of the dye. The dye in the vacuoles is colored from pale lemon-yellow to deep orange-red, indicating a pH range of approximately 6.8 to 8.0. The significance of these changes in color are discussed in a forthcoming paper by Sabin and Doan. It is only in an occasional cell that the differentiation of the lymphocyte from the phagocyte presents any difficulty, such that one might hesitate between calling a cell a heavily stimulated lymphocyte or a small and inactive phagocyte. In general, the larger size of the phagocytes and their dye-filled vacuoles make them one of the most unmistakable cells in the body, as will be readily seen in the illustrations which follow.

[2] Evidence has been presented elsewhere (Kubie and Shults) that this motile lymphocyte is the plasma cell of Unna.

Two morphological groups of phagocytes have been described (Cunningham, Sabin, Sugiyama, and Kindwall; Sabin and Doan; Sabin, Doan, and Cunningham, 1924, 1925) and the subject of their functional and cytogenetic differences has become the object of vigorous inquiry and argument (Lewis and Lewis). One of the groups has been called, after Ranvier's original term, the "clasmatocyte" (Sabin, Doan, and Cunningham, 1924) and the other is the monocyte. Of these two phagocytic forms, only the clasmatocytes are illustrated and discussed here, as they are the characteristic mesodermal phagocytes of the perivascular sheath under these experimental conditions. Monocytes have been seen in only two of the present series of experiments; and since in a previous study (Kubie and Shults), young monocytes were seen to form a normal component of the arachnoid, it is possible that in these two experiments the monocytes may have arisen from bits of arachnoid which were unwittingly included in the preparations.

In Fig. 1, a typical resting clasmatocyte is seen against the endothelial cell of a venule from the occipital cortex of a normal young guinea pig. The cell is stretched along the venule, closely applied to its wall. In other places, as in Fig. 4, it may be seen to stretch along the outer border of the perivascular channel, but in the preparations from normal animals it is always elongated and never rounded up. Even in the normal, however, these cells may be quite abundant. This is shown in Fig. 2, which shows the maximum number of such cells which have been observed in normal material, in this instance taken from the parietal cortex of another young guinea pig.

The contrast between the clasmatocytes of the normal brain and those which show the effect of infection is well illustrated in Fig. 3. Here, the cells appear swollen, often rounded up, with increased vacuoles, and probably an increase in actual numbers. The picture is drawn from an arteriole from the parietal cortex of a young rabbit, at the height of an encephalitis which had been induced by corneal inoculation with herpes virus 6 days before. It will be noted that in this picture two clasmatocytes are seen lying free from the vessel wall. While this is not an unusual observation, it is not the rule; and it is not safe to conclude that the clasmatocytes are able to invade the surrounding brain tissue when in the body, because it is possible that the process of spreading the material could dislocate a few cells, even where the majority have preserved their architectural relationships intact. The clasmatocytes which are occasionally seen free from any vessel wall appear to lie *on* rather than in the ectodermal parenchyma.

In Fig. 5 there is represented the phagocytosis of trypan blue in clasmatocytes along a small vein from deep within the occipital cortex of a young rabbit. This rabbit, over a period of 7 days, had been given three small subarachnoid injections of 1 per cent trypan blue through the cisterna.

Turning to the lymphoblastic group of cells, it was found that small nests of these cells occurred regularly in the normal animals. One such nest is illustrated in Fig. 4, from the cornu ammonis of another normal guinea pig, in the fork formed by the juncture of two venules. Such nests of lymphoblastic cells, containing large, intermediate, and, more rarely, small forms, have been found repeatedly in forks such as the one illustrated. In sections, only a small fragment of such multilayered relationships as these can be demonstrated. Along the unbranched lengths of straight vessels, single lymphocytes, or short chains of three or four in a row, are regularly seen, scattered along the sheath.

The lymphocytic response to disease has been difficult to demonstrate intact, because the larger proliferations are easily ruptured and spill their contents diffusely over the preparation. In Fig. 6, however, is seen the striking multiplication of these lymphoblastic forms in all stages of development. This point of intersection of two venules is from the parietal cortex of an encephalitic rabbit, and at this point it is possible to note a characteristic phenomenon, namely that where the lymphocytic response is intense very little clasmatocytic reaction seems to occur. This is illustrated again in Fig. 5. There is drawn here only one-half of what was actually a long, cigar-shaped dilatation of the vessel sheath. At one end of this fusiform swelling (the end not included in the drawing) were clasmatocytes engorged with trypan blue and quite identical with those pictured at the other pole. In between the two points of intense clasmatocytic response were masses of proliferating lymphocytes which made a cellular tube around the blood vessel, a structure too complex for adequate reproduction. Where the clasmatocytes lie, the sheath is narrow and clings closely around the vessel. Where the lymphocytes proliferate, the sheath is distended, and in it are found only the filmy ends of the clasmatocytes whose main bodies lie at the two poles of the swelling. The stimulation or response of the clasmatocytes and

lymphocytes never seems to occur quite at the same place, but always with a marked preponderance of either one form or the other at any one point.

DISCUSSION.

1. In these studies it has been possible to demonstrate that in the adventitial sheath of the blood vessels of the central nervous system of normal guinea pigs and normal rabbits, both lymphocytes and mesodermal phagocytes are always present, a finding which is opposed to the view of Nissl (pp. 319 and 340), who denied the presence of lymphocytes in normal brains.

The question must be considered as to whether the occurrence of lymphocytes and phagocytes does not from the outset throw doubt on the "normality" of the brains in which they are found, despite the absence of any outspoken evidence of disease. With laboratory-bred rabbits or purchased stock kept under observation, it is always uncertain whether the so-called spontaneous encephalitis exists or not. The condition is usually asymptomatic, and the proof of its occurrence is supplied by postmortem histological examination. Many of the animals used in the present experiments, however, were laboratory-bred and very young. In such animals, these cells have been found just as plentifully in the rabbits and guinea pigs which were under a week old as in the later stages. Furthermore, for the observations on animals more surely normal, most dependence has been placed upon the guinea pig, which almost never suffers from spontaneous encephalitis. And in every animal reported as normal, study of sections from all parts of the central nervous system has revealed no foci of active perivascular infiltration, no neuroglia scar formation, and no changes in the neurons. The absence of histopathological evidence of disease, and the presence of the cells from birth seem to justify the conclusion that the animals were normal.

2. It is at present possible only to suggest the relation which the clasmatocytes of the perivascular sheaths may bear to the microglia of del Rio-Hortega, or to the compound granular corpuscles or *Gitterzellen* of classical neuropathology. If the view is true that an impenetrable barrier separates everywhere the mesodermal from the ectodermal tissues of the brain, then the clasmatocytes cannot be the

phagocytic cells of the ectodermal areas of the nervous system. If, on the other hand, there is no such barrier, it is possible that these cells can wander through the crevices of the brain and perhaps exhibit branching processes which stain with silver and form the pictures of the microglia. To bring final proof or disproof of this possibility will not be easy.

Up to the present time, however, it has not been possible to demonstrate with the supravital technique the existence in the brain of any recognizable mesodermal phagocytes other than these of the perivascular sheath. Neither in the invasive zones described by del Rio-Hortega in the young animal, nor diffusely scattered through the ectodermal parenchyma of the adult, has it been possible thus far to find any cells which react to neutral red in the manner which is characteristic for mesodermal phagocytes of the body as a whole. This negative finding, however, cannot yet be looked upon as conclusive.

3. The normal vacuolation of the perivascular phagocytes is visible in supravital spreads as colorless, homogeneous, refractile globules even in the absence of neutral red. In the presence of the dye the vacuoles are colored from orange-red to pale yellow. This normal vacuolation is lost in the processes of fixation, imbedding, sectioning, and staining. It is usually impossible, for this reason, to differentiate resting clasmatocytes in sections from any other similarly shaped cell, such as an endothelial cell, for instance. In a section, the clasmatocyte and the underlying endothelial cell, drawn in Fig. 1, would usually be indistinguishable. In the living spreads, however, the distinctions are clear. Vacuolation, or other evidence of latent phagocytic power, has never been seen in endothelial cells in these experiments. Even in the necrotic tissue around a cauterized puncture wound, the lining of those vessels which remained intact in that area was unchanged; while the sheath could be seen to have given rise to innumerable phagocytes. The vacuolation of the phagocytic cells constitutes, therefore, a selective stain for these elements in a supravital preparation, enabling one to pick them out with great facility. In sections, on the contrary, it is only when the vacuolation of the cytoplasm becomes greatly intensified, so that the stained cell has a "foamy" appearance, or else when masses of debris have been ingested, that the phagocytic cell becomes definitely recognizable as such.

4. It is noteworthy that the clasmatocytes of the vessel sheaths in the normal brain show this vacuolation. The vacuoles are small in size and limited in numbers, but always present. Early in the course of an herpetic encephalitis they multiply and enlarge greatly, even before fat stains and Nissl stains reveal any marked degree of parenchymatous degeneration. Furthermore, shortly after a sterile puncture has been made in one hemisphere, an increase in the size and number of vacuoles is seen in the perivascular clasmatocytes from distant portions of the opposite hemisphere. This would lead one to suppose that the clasmatocytes are constantly performing some normal function in the central nervous system, of which the vacuolation is an index, and that in the presence of any disease or injury this physiological activity increases before the phagocytosis of debris has commenced.

5. In a recent study of the cerebrospinal fluid (Kubie and Shults), it was shown that the lymphocytes of the perivascular channels are readily extruded into the subarachnoid space. Whether this is also true of the clasmatocytes is uncertain. And how far either of these cells can invade the parenchyma, how extensively they can wander through its crevices along strands of mesodermal tissue, or how strictly they are limited to the perivascular and subarachnoid spaces, are problems which further studies must decide.

6. In the experiments with trypan blue, rabbits were subjected to two or three subarachnoid injections of minute amounts of dye. In each injection, less than half as much trypan blue was administered as fluid withdrawn; and no resisting pressure was encountered. Under these conditions, the trypan blue was found in the clasmatocytes of the vessel sheaths, deep within the substance of certain widely separated regions of the brain. And only in these perivascular sheaths was it found. There was no evidence of generalized diffusion. Furthermore, the dye occurred in the elongated, sessile clasmatocytes alone; and the perivascular channels contained no free wandering cells which could have carried the dye into the depths. The functional patency of these mesenchymal perivascular channels seems to be definitely established by these observations.

SUMMARY.

1. The presence of clasmatocytes in the adventitial sheath of the blood vessels of the normal brain has been shown. Thus far, these are the only mesodermal phagocytes, the presence of which in the central nervous system has proven to be demonstrable by the supravital technique.

2. The clasmatocytes are vacuolated even normally.

3. The vacuolation increases in the clasmatocytes of the whole brain as result of a localized injury, and it increases also throughout the central nervous system in the early stages of an herpetic encephalitis, even before gross damage to nerve cells or myelin can be demonstrated.

4. The endothelial cells of blood vessels have not exhibited any signs of multiplication or of vacuolation. They have not apparently given rise to any of these phagocytes, nor taken any part in the phagocytic reaction themselves.

5. Along capillaries, the phagocytic cells are seen only rarely, and it is possible that they have wandered along these vessels from the nearby venules and arterioles.

6. Trypan blue, injected without pressure into the subarachnoid space, finds its way rapidly down the perivascular sheath and enters the clasmatocytes of the sheath, deep within the substance of the brain. From this fact may be deduced the functional patency of the perivascular channels and their continuity with the subarachnoid space.

7. Lymphocytes are seen normally in the perivascular sheath, and the presence of the large young forms along with the intermediate and the small old cells suggests that they develop in this situation and give rise to the lymphocytes of the cerebrospinal fluid by wandering out into the subarachnoid space.

8. In response to an herpetic encephalitis, as well as other injury, a huge increase in the number of lymphocytes and of clasmatocytes occurs in the perivascular tissues.

9. The multiplication of both cell types seems to begin simultaneously, but never at the same spot, any one point along a vessel exhibiting a marked preponderance of either one form or the other.

10. The resting clasmatocytes cannot readily be differentiated from

any other fusiform cell in sections of fixed tissues; while the lymphocyte may appear, in such sections, as an apparently isolated small round mononuclear cell, with no visible relation to the perivascular apparatus.

CONCLUSION.

By means of the supravital technique, it has been found that the perivascular tissues of the brain contain normally two types of cells—lymphocytes and clasmatocytes. It is believed that these cells are always present and proliferate *in situ*, both under normal and pathological conditions.

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EXPLANATION OF PLATE 24.

FIG. 1. Clasmatocyte against the endothelial cell of a small vein, supravitally stained with neutral red. From the occipital cortex of a normal guinea pig (K 51). × about 500.

FIG. 2. Clasmatocytes stretched along the walls of a branching group of capillaries and venules, supravitally stained in neutral red. From the parietal cortex of a normal guinea pig (K 50). × about 600.

FIG. 3. Clasmatocytes along the walls of arterioles, rounded up in response to the stimulus of an herpetic encephalitis. Supravital staining with neutral red. From parietal cortex of a rabbit (K 53). × about 500.

FIG. 4. Lymphoblastic nest in the perivascular sheath at the junction of two venules, from the cornu ammonis of a normal guinea pig (K 51). Lymphocytes unstained. Clasmatocyte with vacuoles filled with neutral red lies stretched along the outer border of the perivascular sheath. × about 500.

FIG. 5. Reaction of lymphocytes and clasmatocytes to subarachnoid injection of trypan blue. From the occipital cortex of an otherwise normal rabbit (K 74). Trypan blue appears black in the reproduction. × about 500.

FIG. 6. Proliferating lymphocytes at the intersection of two venules from the corpus striatum of a rabbit with herpetic encephalitis (K 68). × about 500.

(Kubie: Perivascular tissues.)

THE RELATION OF MONOCYTES AND CLASMATOCYTES TO EARLY INFECTION IN RABBITS WITH BOVINE TUBERCLE BACILLI.

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PLATE 25.

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In a recent paper, Lewis and Sanderson (1) reported the differences in the reaction in the lungs of rabbits to intravenous injections of human and bovine tubercle bacilli in doses of 1 mg. They studied the material in fixed sections, killing the animals at intervals of 48 hours beginning 2 days after the inoculation. It was found that the response was the same to the two strains of bacteria for the first 8 days, and that then there was a rapid disappearance by absorption of the tubercles in the animals infected with the human strain, in contrast to their continued progressive increase with the bovine organisms. On the 2nd day after infection, they found in all animals a marked thickening of the interalveolar septa of the lungs, due in part to congestion and in part to an increase in mononuclear cells of the "endothelial type," and to polymorphonuclear leucocytes, both amphophilic and eosinophilic. They found small tubercles present from the 2nd to the 4th days.

We have repeated these experiments with rabbits, using the bovine strain of tubercle bacilli only, and making studies of the tissues at intervals of 24 hours for the first 4 days and thereafter every 48 hours throughout the acute reaction of the 1st month. The tissues of the lungs, the liver, the spleen, the mesenteric lymph glands, the omentum, and the bone marrow were studied routinely in the fresh, with the supravital technic, before fixation.

The Lungs.

24 hours after the injection of the bacilli (R 115) no increase in the free cells that come from a gentle scraping of the fresh cut surface of the lung could be

detected. As in the normal, the free cells present were largely of two types, clasmatocytes and monocytes, though there were already present occasional true epithelioid cells, and areas of primitive cells (2) with typical mitochondrial distribution and no reaction to neutral red. Examination of representative areas near the hilum of the lung showed both clasmatocytes and monocytes with the usual phagocytosis of carbon, the former containing it in large masses, the latter, together with an occasional epithelioid cell, storing it in fine scattered granules. While these types of cells were found in relatively the same proportions in other parts of the lungs, notably the apices, the content of carbon was quantitatively less. The polymorphonuclear leucocytes were present in numbers only representative of the circulating blood in the organ.

At 2 days (R 116 and R 215), just as Lewis and Sanderson found, there was an increase in free cells due in large part to clasmatocytes. The preparations all showed an excess of debris and, perhaps correlated with this, an increase of leucocytes. Some primitive cells and monoblasts, many very young monocytes, an occasional epithelioid cell, and lymphocytes were present. At 72 hours (R 120) there were many more small lymphocytes than previously seen, but with clasmatocytes still predominating in the large mononuclear group. From the apex was revealed an area of typical epithelioid cells covering about four oil immersion fields in the microscope, the first appearance of associated epithelioid cells, the earlier reaction being manifested in scattered individual cells only. This beginning formation of tubercles was confirmed in the stained sections of the fixed tissue.

On the 4th day (R 216) the reaction had become predominantly monocytic; many young monocytes were found together with epithelioid cells and typical giant cells of the Langhans type, and certain of the epithelioid cells showed the beginning development of refractile, fat bodies in the periphery. In certain areas the clasmatocytes loaded with massed debris still were very numerous. In supravital surveys, at least, it seems essential, in describing the reaction of the tissues to tubercle bacilli, to differentiate two types of large mononuclear cells. While present together in all preparations, epithelioid cells and clasmatocytes tend to react ultimately so differently to neutral red, their concentration of vacuoles and debris is so dissimilar, and their varying percentages in the course of the development of the special pathology of the disease is so striking, that it is found convenient and necessary to differentiate them in the attempted description of the pathological lesions and their progress. This will be discussed in detail in the latter portion of the paper.

From the 6th to the 18th days (R 217, R 218, R 227, R 228, R 229, R 230, and R 231) the lungs showed an ever increasing limitation of air-containing alveoli coincident with a progressive consolidation with epithelioid and giant cells. Always there were present, however, many young monocytes; at least in one interpretation, the *sine qua non*, the source and origin of the increasing epithelioid cells. Together with the constant presence of the clasmatocytic scavengers

throughout this period, there was evidence of abnormal desquamation of bronchial and alveolar epithelium in the supravital spreads.

The Liver.

No reaction was noted in the liver until the 6th day after intravenous infection, when a few scattered epithelioid cells and one typical giant cell were found in the supravital surveys. The Kupffer cells appeared normal in number and activity. On the 10th day was noted a decided increase in number and activity of the Kupffer cells, some fields showing 18 to 20 cells of this type. There were present many monocytes and occasional epithelioid cells. In one area there was a circumscribed group of 10 to 12 stimulated monocytes looking like a young tubercle. Only limited localizations of epithelioid cells have been observed in the liver of the rabbit under the conditions of this experiment during the 1st month after infection.

The Spleen.

The spleen revealed nothing abnormal in the supravital surveys for the first 72 hours. On the 4th day, however, this organ was definitely enlarged (weight 1.5 gm.), the lymphoid follicles standing out sharply on the slightly bulging cut surface. The clasmatocytes were increased in number; no epithelioid cells were found. On the 6th day the weight was 1.7 gm. On the 8th day there were found primitive cells which were interpreted as monoblasts, and the sections of fixed material on the 6th and 8th days showed a few initial small foci of epithelioid cells. At 10 days, the weight of the spleen was 3.1 gm.; color, deep red; acute splenic tumor, with sinuses engorged with blood. Supravital studies at this time showed increasing monocytes with typical epithelioid cells; clasmatocytes in large numbers contained their usual complement of red cells plus much finer debris. On the 12th day were found scattered everywhere small clumps of epithelioid cells (spleen weight 3.2 gm.) in addition to the clasmatocytes heavily engorged with all kinds of red cell debris. Sinuses were dilated with blood.

The spleen at 14 days weighed 4.9 gm., being dark maroon in color. The clasmatocytes showed marked phagocytosis of both red and white cells, indicating an injury predisposing the latter to ingestion. A few scattered clumps of epithelioid cells were present; sinuses congested.

On the 16th day the spleen weighed 3.5 gm., was a deep red, and presented a moist cut surface. There were from three to five epithelioid cells per oil immersion field and numerous typical giant cells; the lymphoid tissue appeared to be relatively less in amount, some myelocytes were seen, and an occasional large megacaryocyte with massive cytoplasm was observed; the usual clasmatocytes, highly phagocytic, were prominent. On the 18th day the spleen weighed 4.2 gm.

From the 4th day after infection all rabbits surveyed during the 1st month have shown the development of an acute splenic tumor, largely due to dilated

sinuses, increased numbers of actively phagocytic clasmatocytes, and from the 8th to the 10th days increased monoblasts, monocytes, and epithelioid cells, with tubercular foci in limited number scattered both in follicles and parenchyma.

The Lymph Glands.

In the study of the mesenteric lymph glands during the first 18 days after infection, the results have been constant. Beginning with the 4th day these glands have all shown a marked edematous distention due to a great increase of fluid in the lymphatic sinuses. On section of the fresh gland there was a copious flow of opaque fluid having a tendency to coagulate quickly. The supravital preparations were made by taking a drop of this fluid, with and without scraping the cut surface of the gland, the mount being made as with a film of blood. The early reaction as revealed by these preparations was wholly clasmatocytic though only a few intact phagocytes were found. In addition to the usual lymphocytes, mostly small, there were great masses of extracellular stained debris, identical in appearance with that found in the intact clasmatocytes. In conformity with the known fragility of these cells when highly phagocytic, the interpretation that there was great activity of the clasmatocytes or endothelium of the sinuses, and that many of these cells had disintegrated, seems justified (3).

On the 6th day there was found for the first time in addition to the above an extensive stimulation and development of very large young cells, difficult of differentiation between monoblast and lymphoblast at this stage in this environment; many oil immersion fields of the microscope showed as many as 20 such cells. The rabbits representative of the period from the 6th to the 12th days presented much the same picture as just described. On the 12th day, in addition to many young monocytes, typical true epithelioid cells were found, both singly and in small foci, which finding was typical of the glands examined from this date on in the acute period of the disease.

These observations agree in general with those of Kageyama (4) who made studies of the lymph glands of mice after intraperitoneal inoculation. He found the tubercle bacilli free in the sinuses after 5 minutes, and within the lining endothelial cells in 15 minutes. These endothelial cells rounded up and became free and within 2 hours could be shown to be filled with bacilli. The cells died in from 6 to 18 hours, the leucocytes then coming into activity. The leucocytes in turn died and were phagocytized by new histiocytes; after the disintegration again of the latter no bacilli could be stained. Only after the 7th day were the epithelioid cells of tubercles found, containing very few bacilli.

These combined observations indicate the marked phagocytic activity of the clasmatocytes for tubercle bacilli, the fragility of the cells, but their apparent power in at least destroying the acid-fast characteristic of the organism. Also, in contrast to the lung, the late development of tubercles in lymph glands is indirect evidence against the normal presence of monocytic foci in the latter.

The Omentum.

The omentum lends itself admirably to supravital surveys, because the relationships of all cells are maintained in the thin films of this tissue, which it is possible to make on slides prepared in the usual manner. Until the 4th day the omentum showed only the usual clasmatocytic and fibroblastic distribution of cells. On the 4th day there appeared in addition a few, scattered, young, developing monocytes, and the clasmatocytes from this stage on showed increased phagocytic activity and were predominant in their numerical strength up to 12 days. On the 8th day many clasmatocytes in the omentum contained acid-fast debris. On the 14th day epithelioids had appeared among the increasing monocytes and definite aggregations of epithelioid cells in tubercles were found on the 16th day.

The Bone Marrow.

The reaction of the bone marrow is initiated on the 8th to 10th days by the development of large numbers of young monocytes *in situ*, followed from the 12th to the 20th days by an increasing encroachment on fat cells and hemopoietic foci by typical tubercular tissue. This process is followed in detail in an earlier paper in this *Journal* (5).

Thus it will be seen that with the intravenous portal of entry the initiation of the tubercular process in the lungs definitely antedates that observed in the other organs of the body as analyzed in these supravital surveys and confirmed by study of fixed sections. The first epithelioid cells are found in the lung within 24 hours after the injection of tubercle bacilli but not until the 6th to the 14th days in the other tissues studied. The earliest and most obvious reaction in all organs is the clasmatocytic response, and the development of the tubercular tissue, epithelioid and giant cells, is always preceded or accompanied by a stimulation of monoblasts and young monocytes, apparently *in situ*.

There is at present much difference of opinion concerning the phagocytic mononuclear cells, the prevailing idea being that they constitute a single strain. The recent studies by the method of tissue culture by Carrel and Ebeling (6), and by Lewis and Lewis (7) have brought strong support to this view.

Carrel and Ebeling studying monocytes from the blood and macrophages from the connective tissues found that the monocytes taken directly from the blood of the animal were smaller than the macrophages but that this distinction disappeared with proper nourishment in tissue culture; that monocytes and macrophages grew well with the same nutrient elements in contrast to fibroblasts; that they were

equally affected by certain toxic agents; and that they had the same type of actively waving surface films. From these common properties they have concluded that any morphological differences between the two types are due to environmental influences. Lewis and Lewis have made extensive comparative studies of blood beginning with the fish and have also concluded that monocytes and clasmatocytes are a common strain. Neither of these studies with tissue culture has taken into consideration the fact that the desquamated endothelial cell, often a degenerating type it is true, but also quite as often a living, active cell, is present together with the monocyte in normal blood (8). Moreover, even as blood cannot be considered the source of a pure strain of monocytes, neither can the connective tissues be considered the source of a pure strain of clasmatocytes since both clasmatocytes and monocytes are present in connective tissues. However, these experiments with tissue culture are a great advance in our knowledge of the phagocytic mononuclear cells. Though there is considerable evidence to show that these two strains of cells do vary independently in their response to certain pathological conditions, nevertheless Carrel and Ebeling have definitely proved that under the conditions of tissue culture they both respond alike to certain stimuli.

The history of the type of cell now discriminated as the monocyte, distinct on the one hand from the granulocyte and on the other from the lymphocyte, is a confused one. The monocyte was first considered in the Ehrlich classification as a transition form in the maturation of the leucocyte. This error involved the idea that the specific granulation of the monocyte was of the neutrophilic type. The correction came with the introduction of the Romanowski methylene blue-azur, specifically with the use of azur. With this technic, it was discovered by Michaelis and Wolff (9) that both lymphocytes and monocytes (transitional leucocytes) contained azur granules. That the granulation of the monocyte has no relation to the neutrophilic granulation was fully demonstrated by Pappenheim and Ferrata in 1911 (10). The monocyte was described as making a third strain of the white cells of the blood, distinct from leucocytes and lymphocytes, by Schilling-Torgau in 1912 and 1914 (11), and since then this interpretation has been generally recognized and accepted by hematologists.

However, the literature, particularly the clinical, has continued, even to the present time, to give records of blood counts in which monocytes and lymphocytes are recorded together as mononuclear cells without differentiation. The confusion attendant upon the lack of recognition of these cell differences clinically may well be illustrated in the disease known as infectious mononucleosis. Unpublished observations by both of the present writers in independent studies at the Johns Hopkins Hospital and the Boston City Hospital confirm the lymphocytic, rather than the monocytic, nature of the mononuclear increases in this condition.

The discrimination of lymphocytes and monocytes can be made in a good methylene blue-azur stain. The cytoplasm of the monocyte reacts with a dense muddy blue color in which are embedded in the human strain varying numbers of tiny azurophilic granules (12), not identical with the azur granules of lymphocytes, since they are smaller and stain darker. The clearness with which these tiny granules may be seen is a good gauge of the success of the stain and the adequacy of the lens used. If the stain be too basophilic, the granules will be obscured.

In the fourth edition of his text-book, Naegeli (13) states that these granules of the monocyte can be seen with dark-field illumination in the living cell. This we have confirmed. Since the monocyte has great numbers of tiny mitochondria, however, it is necessary to identify them also with the dark-field illumination and to discriminate them from the specific granulation. If a film of blood be stained with neutral red and Janus green and studied with a microscope in which first direct and then dark-field illumination can be applied to the same cell, it is easy to demonstrate with a lymphocyte that the mitochondria, stained green, but refracting pink in dark-field illumination, are the only bodies visible. By selecting a monocyte in which there are but few neutral red vacuoles and in which the mitochondria are well stained, the cytoplasm of the human monocyte under the dark-field appears fully as granular as the neutrophilic and the eosinophilic leucocytes themselves. This granulation, seen with the dark-field, in the human monocyte is neither the same substance as the bodies that react to neutral red in the living cell nor the mitochondria, and is, therefore, by one more criterion differentiated

from the myeloid granulation. In the monocyte of the rabbit, there is a marked reduction of the azurophilic granulation in fixed films, most of the cells showing none at all, and the granulation visible in dark-field illumination suffers a corresponding reduction. In a more recent review, Naegeli (12) gives a plate of human monocytes showing the variations in the content of azurophil granules down to a cell, representative of the occasional human monocyte, which lacks them entirely. The latter is entirely comparable with the usual monocyte of rabbit blood, the basic muddy blue cytoplasm typical of all cells of this type being independent of species and granulation. With reference to this strain of cells in the two species, the peroxidase reaction has been found (14) to parallel the azurophilic granulation qualitatively and quantitatively, the majority of human monocytes giving a positive oxidase reaction, the opposite being true of the rabbit monocyte.

An adequate and assured discrimination between lymphocytes and monocytes became much easier with the introduction of the supravital technic for the study of blood (15). When attention was focused on living blood cells studied in their reactions to neutral red and Janus green, distinctions between these two strains of cells, other than those above mentioned, became clear. The lymphocyte is characterized by a clear cytoplasm in which are many mitochondria and a few scattered vacuoles, which stain in neutral red, and which vary greatly in number and size according to the activity of the cell; these vacuoles in fresh preparations of normal blood are often entirely absent in the small forms. The nucleus is seldom round, but changes constantly in shape, especially as the cell moves. The type of motility is characteristic, and like that seen in no other cell; the nucleus is always in the front end of the moving cell and changes so rapidly in shape that it seems to take an active part in the locomotion of the cell.

The monocyte, on the other hand, both of human and of rabbit blood, shows a rosette of neutral red bodies of a uniform salmon tint, and great numbers of tiny mitochondria. The color and nature of the vacuoles reacting to neutral red are identical in all species, their relative number only influencing the sharpness of definition of the clearly maintained centrosphere. In the specific reaction to tubercular infection, the epithelioid and giant cells of rabbit and human

tissues are indistinguishable in their characteristic reaction to vital dyes, while obeying the species response to the peroxidase reaction (14); rabbit epithelioid, oxidase-negative, human epithelioid, oxidase-positive. The cells in locomotion are stretched out often in triangular shape; there is a slow progression with less active streaming of particles than with the granulocytes; in the supravital technic, the surface films do not have as free play, due to the narrowness of the space between slide and cover-slip, as in the tissue culture preparations. These distinctions of motility are fully demonstrated in the moving pictures of Carrel, Ebeling, and Rosenberger.

Thus, in the history of the study of the blood the monocyte was first confused with the neutrophilic leucocyte, this error being gradually corrected when it became clear that the monocyte never developed a neutrophilic granulation. The monocyte then came to be regarded as indistinguishable from the lymphocyte. But the azur granules of lymphocytes are large, few in number, and inconstant, while the azurophilic granules of the human monocyte are tiny and usually extremely numerous; the degree of basophilia of the cytoplasm is distinguishing; and the living cells show a different type of motility and react characteristically to the supravital dyes. Then, too, the differential response in pathological states has further separated the types (16).

We are now in a third stage in the history of the monocyte in its struggle for recognition as an entity, when it is by some regarded as identical with the clasmatocyte of the connective tissues. It is clear that in the present day study of the mononuclear cells, the first step, as in the past, must be the effort to see if there are finer morphological discriminations by which different types may be distinguished, and, then, to see if such criteria may be of aid in finding out and analyzing any differential response of these cells in physiological and pathological conditions.

The morphology of a cell with such a wide range of phagocytic activity as the clasmatocyte must be varied. The cytoplasm of the clasmatocyte stained in methylene blue appears as a faintly basophilic thin film (8) in contrast to the dense cytoplasm of the monocytic strain. This contrast is shown in Figs. 8 and 9, which are clasmatocytes, in contrast to Figs. 6 and 7, which are an epithelioid cell and

a giant cell of the Langhans type. The cytoplasm of the clasmatocyte has no specific granulation except for substances phagocytized. Hence, when granules are present, they may be of any size from tiny points to whole included cells, and they may react with any of the shades of the dyes used, according to the original nature of the substances taken in or the state of digestion in which they are found. Likewise, in the supravital technic, the clasmatocytes vary all the way from a small cell with a few vacuoles stained with the neutral red up to enormous cells engorged with whole cells and debris.

In general, there are two groups of clasmatocytes: first, the constantly active phagocyte of certain organs, the derivatives of the so called specific endothelia, namely of spleen, lymph glands, bone marrow, and liver; and secondly, the normally less active phagocytes of the general connective tissues.

With the supravital technic we have found that the normal monocyte of the blood and the resting clasmatocyte of the tissues (Fig. 3) are readily discriminated by the nature of the stained vacuoles. This much might be granted under either concept of their relationship, whether they are to be considered as different strains or simply as different phases of the same strain of cells. The vacuoles of the monocyte of the blood show a uniform salmon-red reaction to vital neutral red and they form a relatively permanent rosette around the centrosphere of the cell.

In the unstimulated clasmatocyte of the tissues, the vacuoles are not in a rosette but are scattered throughout the cytoplasm, and they may stain red, or orange, or yellow; moreover when the cell is watched in the supravital preparation, there is usually a tendency for the color, if originally red, to change toward yellow. When the monocyte is watched in the supravital film, the red color of the vacuoles tends to be retained persistently even to a stage of degeneration which involves a destruction of the cell membrane. This phenomenon is not seen unless the dye is in a concentration that is differential.

A dilution of vital neutral red to be discriminatory must be carefully adjusted. Fortunately, in normal blood, there is an excellent standard for the optimum range of neutral red as an indicator; the eosinophilic granules should show the alkaline or yellow reaction of one extreme, pH 8; the basophilic granules should show the other

extreme with the deep scarlet of the acid end, pH 6.8; the vacuoles of the monocytes should give the salmon shade of an intermediate pH. In rabbit blood with the high percentage of basophils and sufficient eosinophils, the standardization is readily made. Most of the neutral red in use gives a yellow tone modified by a coppery tinge for the eosinophilic granules. A perfect dye has only the yellow tone. Occasionally two different eosinophils in a drop of blood vary slightly in their reaction to the dye. The basophils must have the brilliant scarlet reaction. If the concentration of the neutral red is too great, every substance that reacts at all in the cells will be a deep uniform red. Usually in such a preparation a lessened motility of the cells will also indicate a toxic reaction of the dye. In a dilution that brings out the entire range of color from red to pure yellow, the vacuoles of the monocyte and epithelioid cell are distinctly and uniformly within the acid range of the dye but never as sharply scarlet as the reaction of the basophilic granules. We have described the characteristic shade of the vacuoles of the monocyte as a salmon-red. In becoming the epithelioid cell the vacuoles may assume a uniform carmine tone, as is shown in Fig. 2. The clasmatocyte on the other hand quite as characteristically shows every range of color of the dye in the same cell, often all shades being present. The clasmatocyte of Fig. 5 shows the orange and yellow tones.

Because the shade of the dye in the vacuoles of digestion of the clasmatocyte frequently changes from the varying tones of red through orange to pure yellow while the cell is watched, the question is raised as to whether this change may not represent a physiological process fundamental to this type of cell, indicating that the development of a relatively alkaline reaction may be essential to the digestive processes of the vacuoles. On the other hand, this change in color may be only the less significant step in a process of degeneration and cell death. If the latter be true, then monocytes do not tend to degenerate in this manner.

Often the clasmatocytes show the yellow reaction of the vacuoles when first taken from the animal. This was conspicuously the case with the stimulated clasmatocytes of the lung the first 3 days after the injection of tubercle bacilli. These contrasts are well seen in cells of Figs. 1 to 5. Figs. 1, 2, and 3 are a fibroblast, an epithelioid

cell, and a clasmatocyte taken from the omentum of a rabbit, R 160, which had received twelve doses of a phospholipin, designated A-3, isolated from human tubercle bacilli by Dr. R. J. Anderson. The full account of the experiment is given in an accompanying paper in this *Journal* (3). The three cells, Figs. 1, 2, and 3, were in the same field; the fibroblast was in the middle and the other two were not more than the diameter of two red cells apart. The red cell of Fig. 4 gives the magnification. The fibroblast was unstimulated and showed no reaction to the neutral red. The epithelioid cell of Fig. 2 is typical; it is the type of cell characteristic of the lesion of tuberculosis itself, in this instance produced in response to a chemical factor from the bacillus. It shows the carmine reaction of the tiny vacuoles of the rosette and this color showed no tendency to fade on standing. The clasmatocyte, Fig. 3, is the typical long cell characteristic of the resting type to be seen in the normal omentum and in the general connective tissues. When this cell was first seen, its vacuoles had considerable red but the epithelioid cell was drawn first and by that time all the vacuoles of the clasmatocyte were yellow as shown in the drawing and yet there was no indication of any damage to the cell. The omentum provides perhaps the best material for supravital studies in the early stages of a peritoneal irritation, because it is so thin a film that one can, so to speak, dissect the entire thickness with the focusing screw of the microscope, and because the cells are retained in their original relative positions. The position of these three cells, all within one oil immersion field of the microscope and all three showing a different reaction to an identical concentration of the same dye, is evidence toward the view of a differentiation of the cells of the connective tissues in an environment that must be essentially the same for each. It is difficult to imagine any great difference in environment between these three cells as far as soluble factors are concerned. The phospholipins of the experiment were not in solution but in the form of an emulsion, but there is evidence that the clasmatocytes also take in lipoids. If, then, both types of cells take in the lipoids, it appears that they treat this material differently.

The cell of Fig. 5 was in our interpretation a clasmatocyte from the circulating blood of a rabbit, R 193. This animal was one of a group of six animals which were inoculated with 1 mg. of a culture

of Strain B1, which had been growing for 2 months and proved to be largely attenuated. The signs of tuberculosis were slow in developing; there was no anemia in a month, and in only two of the animals was there any increase in monocytes in the blood. The constant reaction of the blood during the 1st month to an active culture of this strain (B1) has been reported in another communication (5). A second dose of 2 mg. from a fresh culture of the same strain of bacilli was given 1 month after the first injection. The next morning five of the six animals were dead; two of them were still warm so the living cells could be studied, and the reaction in all the tissues with the supravital technic was of the clasmatocytic strain. In the sixth animal, that survived, there was a rise in clasmatocytes to 1600 per c.mm. on the next day, there were 1200 cells too far degenerated to be classified, while the monocytes showed only 800 per c.mm. of blood. The cell of Fig. 5 was drawn during this rise (see Chart 5 of a preceding paper (5)). It will be noted that the cell was heavily vacuolated with orange as the predominating shade, and during all the time that the cell was being drawn the color was changing toward the yellow tones. This slide was kept for 3 to 4 hours and the monocytes still retained their red vacuoles long after all color had faded from the clasmatocytes and they had disintegrated. We have discussed elsewhere (8) the fragility of the clasmatocytes in supravital preparations and their tendency to a sudden bleaching of all color at the time of the death of the cell. In this series of experiments, every lymph gland studied in the acute reaction to a massive injection of tubercle bacilli showed debris which had probably come from the disintegration of clasmatocytes. The debris found in fresh scrapings of the lungs 2 days after inoculation may have been from the same source since the only marked increase in cells at that time was in the clasmatocytic strain. An excess of cellular debris is also frequently found in the peripheral blood of the rabbits during tuberculosis.

In the reaction to neutral red, the monocyte shows an almost uniform color of the vacuoles in a given cell and the shade is of a salmon tint which may become a carmine tone in the tiny vacuoles of the epithelioid cell. The color of the vacuoles of the clasmatocyte varies all the way from red to yellow in one cell, and there is every possible combination of shades with marked changing toward yellow in many

cells. It is clear, then, that while there is an overlap in color reaction of these two strains toward the acid range of the dye, only the clasmatocyte tends toward an ultimate alkaline reaction.

It has been brought out by Lewis (17) that the arrangement of vacuoles in a rosette around the centrosphere can be observed in the clasmatocyte as well as in the monocyte. This is certainly true. It is also well known that at a certain stage in the development of the granulocytes, the neutrophilic, eosinophilic, and basophilic granules are arranged so as to accentuate the centrosphere. This is, however, a transient stage in the development of the leucocyte but this phase will be seen in the blood whenever conditions bring slightly less mature leucocytes from the bone marrow. In studying living cells taken directly from the animal, a rosette formation is the exception for the clasmatocyte and the rule for the monocyte. Moreover, in the epithelioid cell of the tubercular process, the rosette formation of the neutral red bodies becomes the constant, chief morphological characteristic of the cell.

In an earlier paper in this *Journal* (5), we recorded the finding of clasmatocytes in the bone marrow filled with acid-fast debris as the epithelioid cells of the local tubercular process degenerated spontaneously. These cells were of the type of the branched adventitial cell of Marchand, stretched along the vessels so that of their identity with the clasmatocyte or macrophage strain there was no question. In Figs. 6 to 9 is shown the contrast between the reaction of the clasmatocytic and the monocytic strains to tubercle bacilli as stained by the Ziehl-Neelsen technic. Figs. 6 and 7 are of the monocytic strain. Figs. 8 and 9 are clasmatocytes. Figs. 6, 7, and 8 are drawings made with the camera lucida of cells from a scraping from the lung of a rabbit (R 216) 4 days after the injection of tubercle bacilli. All three were taken from adjacent fields in the same preparation. Fig. 6 is a young epithelioid cell containing seven intact tubercle bacilli. The cytoplasm stains a deep blue with the methylene blue of the counterstain and is exactly like the reaction of the cytoplasm of the giant cell, Fig. 7. In marked contrast is the pale, foamy, and much vacuolated cytoplasm of the clasmatocyte, Fig. 8, with one large round nucleus. In the giant cell there are at least three nuclei, and the cell is filled with great numbers of intact tubercle bacilli.

In the two clasmatocytes, on the other hand, Fig. 8, from the lung, and Fig. 9, from the omentum, of another rabbit of the same series (R 218) killed 8 days after the injection of the bacilli, the reaction is entirely different. The cell of Fig. 9 is the same type as the long clasmatocyte from the omentum shown on the same plate as Fig. 3. In both Figs. 8 and 9 are shown acid-fast fragments, the cell of Fig. 8 being the key because it has tubercle bacilli apparently in the process of fragmentation. In the omentum from which the cell of Fig. 9 was drawn, it was not simply the occasional clasmatocyte which showed this reaction, but there were wide zones in which every clasmatocyte contained the acid-fast debris. Likewise, in the sections for bone marrow which have shown this reaction, it has not been the occasional clasmatocyte which contained the specific debris, but numerous cells throughout the section.

The occurrence of acid-fast granules in tubercular tissue is well known to the pathologist though it is not regarded as sufficient evidence for a diagnosis of the disease in the absence of, or in the failure to find, the actual intact bacilli, but we are bringing evidence to show that they may be significant in the analysis of how the organism deals with a tubercular infection. Our observations indicate that there is a certain difference in the response of two closely allied strains of mononuclear cells to this specific pathological agent; that, while both clasmatocytes and monocytes are, under appropriate conditions, highly phagocytic types, the one form tends to break up tubercle bacilli and the other type retains them intact and alive with reproductive multiplication for long periods of time.

In these studies it appears that the immediate major response of the connective tissues to the infection of tuberculosis involves two strains of cells, clasmatocytes and monocytes. The more chronic response of the lymphocytes is well known through the work of Murphy (18). The clasmatocytes are the macrophages of the general connective tissues and respond to the presence of large numbers of tubercle bacilli as to any other debris or foreign material, phagocytizing and fragmenting with the attempt to destroy vast numbers of the bacilli. There is evidence that in this process many clasmatocytes are themselves destroyed. During an infection with tuberculosis

great numbers of clasmatocytes containing acid-fast debris may be found in the tissues.

With the monocyte, on the other hand, the infection exerts an influence whereby this cell becomes the typical epithelioid type, eventually making the characteristic lesion of the disease; in the epithelioid and giant cells the tubercle bacilli remain intact and apparently survive over long periods of time. The epithelioid cell appears in the lung within 24 hours after the injection of the bacilli, which is correlated perhaps with the observation that in the lung normally at all times are to be found numbers of mature monocytes. In other organs, this normal presence of monocytes is limited, and may represent one of the factors in organ susceptibility; the normal number of monocytes or the degree to which monoblasts may be stimulated to development and maturation, together with the activity of the clasmatocytes in destroying bacilli, in any particular region would appear to be a function of the rapidity and extent of the local tubercular involvement.

SUMMARY.

1. The early reaction to intravenous tubercular infection in the various organs of the rabbit reveals a pathognomonic response in the lungs within 24 hours; the specific response in the liver, spleen, lymph glands, and bone marrow, follows from the 6th to the 14th days.

2. The development and extent of the pathologic process has been analyzed in terms of the activity of monocytes and clasmatocytes.

3. The criteria for differentiating these mononuclear phagocytic cells into two strains have been analyzed and the technics discussed.

4. The clasmatocyte phagocytizes tubercle bacilli freely and fragments them, as it does all cellular and other debris.

5. The monocyte stimulated to metamorphose into the typical epithelioid and giant cell of the Langhans type retains the tubercle bacilli intact, with power to survive and multiply, over long periods of time.

6. The normal number of monocytes or the degree to which monoblasts may be stimulated to development and maturation, together with the activity of the clasmatocytes in destroying bacilli, in any

particular region, would appear to be a function of the rapidity and extent of the local tubercular involvement.

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EXPLANATION OF PLATE 25.

FIG. 1. Fibroblast from the omentum of a rabbit, R 160, which had received ten intraperitoneal doses of 80 mg. and two of 160 mg. of a phospholipin designated A-3, isolated by Dr. R. J. Anderson from human tubercle bacilli, Strain H 37. The peripheral blood from a similar experiment is shown on Chart 4 (R 153) in a paper in this *Journal* (3). The cells of Figs. 1 to 4 were from the same preparation and were all within the same oil immersion field. Drawn from the living cell stained supravitally with neutral red. The magnification is shown by the red cell of Fig. 4

FIG. 2. Epithelioid cell from the same preparation as Fig. 1. The color of the reaction of the rosette to neutral red, namely carmine, was carefully matched.

FIG. 3. Resting clasmatocyte from the same preparation as Figs. 1 and 2. The vacuoles in the preparation when first seen had some red tones of the dye, but were all yellow, as shown, by the time the epithelioid cell of Fig. 2 had been drawn.

FIG. 4. Red cell for the magnification of Figs. 1, 2, 3, and 5.

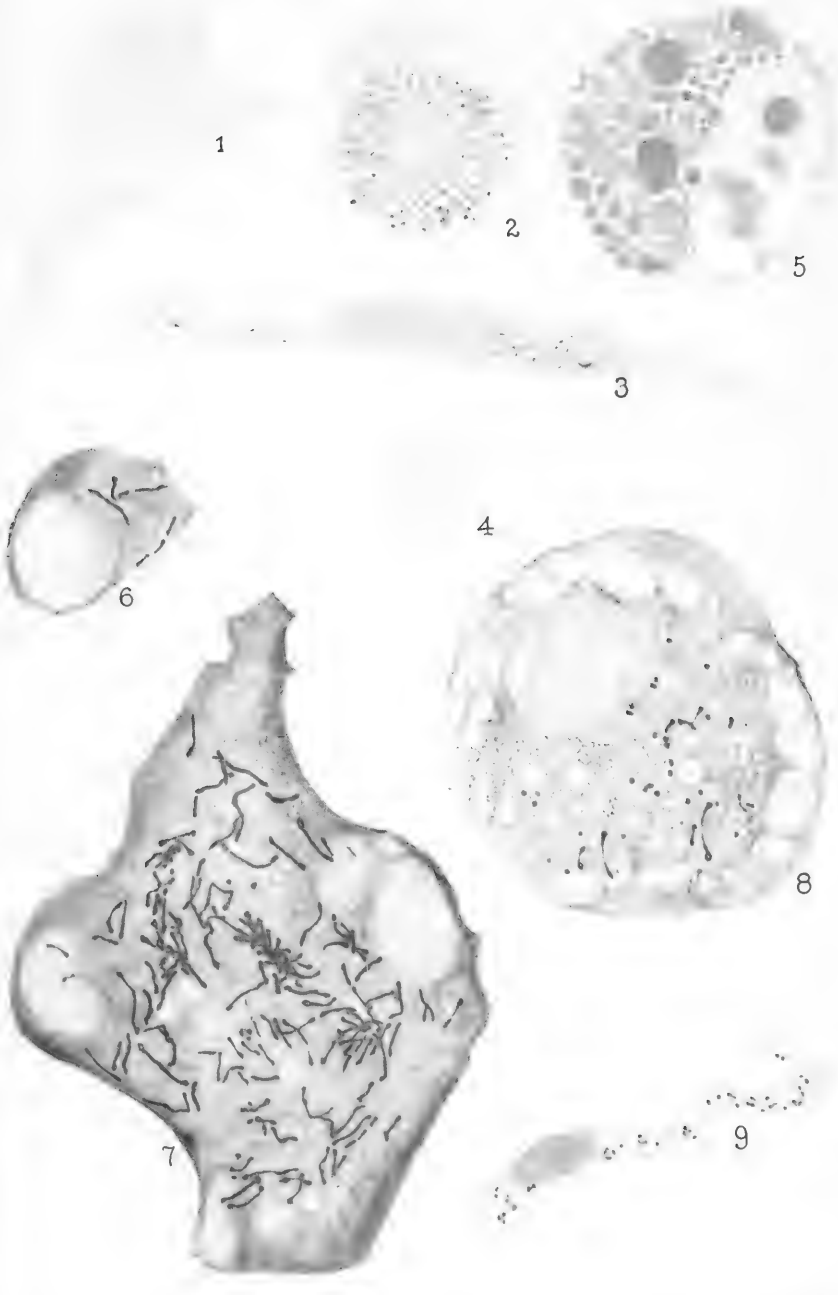
FIG. 5. Clasmatocyte from the peripheral blood of a rabbit, R 193. The studies of the blood of this animal are shown on Chart 5, in a paper in this *Journal* (5). The cell was drawn on March 22, 1927, 24 hours after the second intravenous injection of bovine tubercle bacilli, when there were 16 per cent clasmatocytes, 12 per cent degenerating cells, and 8 per cent monocytes in the circulating blood. Drawn from the living cell stained with vital neutral red. The magnification is about the same as that of Figs. 1, 2, and 3.

FIG. 6. Epithelioid cell with seven intact tubercle bacilli from a scraping of the cut surface of the lung of a rabbit, R 216, 4 days after an intravenous injection of 5 mg. of living tubercle bacilli. Stained with Ziehl-Neelsen technic for tubercle bacilli. The cells of Figs. 6, 7, and 8 are all from the same slide; were drawn with the camera lucida, magnification 1700, with the reaction to the methylene blue carefully matched.

FIG. 7. Giant cell of the Langhans type from the lung, showing many intact tubercle bacilli. Drawn from the same slide as the cell of Fig. 6.

FIG. 8. Clasmatocyte from the lung, showing many tubercle bacilli in the process of fragmentation. From the same preparation as Figs. 6 and 7.

FIG. 9. Clasmatocyte from the omentum of a rabbit, R 218, 8 days after an intravenous injection of 5 mg. of living tubercle bacilli. Fixed in formalin and stained with Ziehl-Neelsen technic for tubercle bacilli. Drawn with the camera lucida at a magnification of 1700. This cell was one of many in the preparation to show acid-fast debris.



(Sabin and Doan: Infection with bovine tubercle bacilli.)

THE BIOLOGICAL REACTIONS IN RABBITS TO THE PROTEIN AND PHOSPHATIDE FRACTIONS FROM THE CHEMICAL ANALYSIS OF HUMAN TUBERCLE BACILLI.

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Plates 26 to 29.

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Through the organization of the Research Committee of the National Tuberculosis Association, for cooperative research, we have had available for biological testing certain protein and phosphatide fractions isolated in the chemical analysis of tubercle bacilli. Two specimens of water-soluble proteins, designated 304 and 903 respectively, were received from Professor Treat B. Johnson of the Sterling Chemistry Laboratory of Yale University, and two phosphatide fractions, A-3 and A-4, from Dr. R. J. Anderson, of the same laboratory (1, 2). The material was obtained from the analysis of human tubercle bacilli, strain H 37.

In another paper in this *Journal* (3) evidence has been given to show that clasmatocytes and monoytes react differently toward tubercle bacilli. By some investigators, particularly those using the technic of tissue culture, it is believed that this phagocytic mononuclear group represent essentially a single strain of cells, variations in the physiological environment being thought sufficient to account for differences in the histological structure within the limits observed. However, an increasing weight of evidence accumulating as a result of the direct analysis of the response of the cells of the connective tissues *in situ* to pathological conditions, is necessitating the subdivision of this group on functional, as well as genetical and structural (4, 5) grounds. For example, while both clasmatocytes and monocytes phagocytize tubercle bacilli, the clasmatocyte on the one hand fragments the bacteria readily and rapidly as it does all debris with

which it has to deal; the monocyte, on the other hand, retains the specific bacillus intact so long that the relationship may be considered one of symbiosis.

And now, again, in analyzing the responses of tissue to the protein and phosphatide fractions from the tubercle bacilli, it has been found necessary to express the differential reactions in terms of the same two types of cells within this mononuclear phagocytic group: the response to the protein fractions has been predominantly clasmatocytic, in sharp contrast to the development of monocytes, epithelioid cells, and Langhans giant cells, making the typical lesions of tuberculosis, which is the overwhelming reaction to the phosphatide fractions.

The amount of material available limited the administration of the protein fractions to seven rabbits, the phosphatide fractions to five rabbits, with a control group consisting of six rabbits.

The Protein Fractions.

Both the proteins, 304 and 903, were given intravenously from saturated solutions made up each day in water freshly distilled from glass. From a preliminary experiment (6) it was known that rabbits could readily withstand a dosage of 10 cc. of the saturated aqueous solution of 304. With this experience as a basis a similar dosage of 903 was given to Rabbit R 138, with an immediately fatal result simulating anaphylactic shock. A second rabbit, R 132, survived the initial dose of 10 cc. of 903, but died immediately after a second dose 24 hours later. In the latter animal the white count before the first injection was 9350; on the next day, just before the second inoculation, the white cells had risen to 25,000. At autopsy the lungs showed hemorrhagic foci and the free cells were predominantly clasmatocytes and leucocytes. The protein fraction 903 was, thus, found to be far more toxic than 304, since in the present experiment two rabbits survived ten and eleven doses, respectively, of the latter in the dosage that proved fatal with 903.

To determine the upper limit of sublethal dosage for 304, Rabbit R 152 was given two injections of 20 cc. each at an interval of 24 hours. The studies of the peripheral blood of this animal are shown on Chart 1, the data concerning the white cells being given on a scale one-half the magnitude of the other graphs to cover the rise in leucocytes. The dosage of 20 cc., while not lethal, was too toxic to study other than the acute reaction; the day after the first injection the animal appeared ill, ate but little, and showed a rectal temperature of 107.6°F. (normal 102–104°F.); following the second dose the condition did not warrant further injections. From the chart it is obvious that the animal was developing an anemia, the red cells

falling from 6,800,000 to 4,100,000 and the hemoglobin from 65 to 53 per cent; while the white cells mounted from 10,000 to 27,000. On the two counts made before the injection, the neutrophilic leucocytes were low, 38 and 29 per cent, as can be seen on the chart by a comparison of their number and the total number of the white cells. However, the total myeloid cells were about half the white cells, 54 and 57 per cent, due to the relatively large percentage of basophilic cells, which were 13 and 22 per cent in respective counts. 24 hours after the first injection a marked rise in the total number of white cells may be seen to have been due wholly to an increase in neutrophilic leucocytes and this continued to be true throughout the experiment. The basophilic and eosinophilic leucocytes, on the other hand, remained unaffected at their original levels indicating a specific stimulating effect on one group of granulocytes only, namely the neutrophils. There were interesting qualitative as well as quantitative changes in the neutrophilic leucocytes: on Jan. 4, of the 67 per cent neutrophils, 57 per cent were of the non-motile (7) type. These are the forms that appear as ruptured cells with scattered granules in fixed films, showing that they are fragile. 24 hours after a second dose of 304 was given, the white cells showed some further increase but now there were found two types of neutrophils, old, degenerating types with fragmenting nuclei and a new group of peculiar young forms. These young leucocytes had indented nuclei, not yet two lobed; they were markedly deficient in the specific granulation and rich in mitochondria; and they were not actively ameboid. In fixed films these cells had the other characteristic of incomplete maturation, namely a strongly basophilic cytoplasm. At this examination the leucocytes were about equally divided between the old forms and the young. A second count, in the afternoon, showed a fall in total number of white cells to within normal limits with only 6 per cent of the degenerating leucocytes remaining. On the next day the total white count had risen again to 27,000 and the young leucocytes as described were the predominating type. As will be seen on the chart, both monocytes and clasmatocytes rose slightly while the lymphocytes remained at the low level to which they had fallen after the first dose.

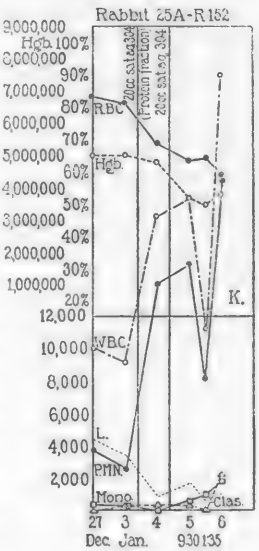

CHART 1.

The studies of bone marrow following the autopsy on the 4th day revealed something of the nature of the processes already indicated in the studies of the peripheral blood. The bone marrow had been depleted rapidly of its store of mature myelocytes, Type C (8): this was evident in the supravital preparations of the fresh marrow and in sections. The sections showed that the interspaces

between the fat cells had been denuded of cells so that they were like the sections of marrow shown by Doan *et al.* (9) both from experimental animals treated with repeated doses of dead typhoid bacilli and from a human case of typhoid fever. In the supravital preparations of this marrow the immature myelocytes, Type B, with fewer of the specific ganulations, showed a premature indication of polymorphism of the nuclei and the beginning of motility. Thus the leucocytes of the blood stream had had certain of the characteristics of the B myelocytes, namely the basophilic cytoplasm, the large content of mitochondria, and the low content of specific granules; while their nuclei were, on the other hand, of the type of the metamyelocyte, that is indented but not yet lobed. The nuclei showed that the cells were being transformed into functionally active leucocytes without complete maturation relative to their full quota of granules. These cells therefore were entirely different from the immature and unchanged B myelocytes that occur in the blood stream in leucemia (8). This type of cell, the immature but motile leucocyte, is especially interesting because it occurs in the blood in typhoid fever (10), probably for the same reason as in this rabbit with the massive doses of protein. It must be discriminated with care from the monocyte both in the fixed films and in the supravital technic. The discrimination is to be made by the nature of the granulations. In this rabbit, it is interesting to note that the effect of the protein was limited to the neutrophilic (pseudo-eosinophilic) leucocytes, both the basophilic and the eosinophilic cells being unchanged in the blood and in the bone marrow; the reaction thus represents a response to a protein in one strain of granulocytes. This type of reaction is of course not specific of a protein from tubercle bacilli only.

The autopsy showed a general increase in clasmatocytes in the tissues in the lung; as determined in supravital studies, many of these clasmatocytes contained fragments of red cells. In sections the interalveolar septa were thickened and a few foreign body giant cells were found. As in all the animals with the protein fractions, there were hemorrhages. In this animal they were especially marked in the bone marrow as shown by sections. There were gross hemorrhages in the retroperitoneal tissues extending into the psoas muscle on either side.

The liver showed changes. There were certain chronic periportal lesions entirely independent of the experiment. The acute lesions of the liver were irregular, small zones of necrosis, involving 20 to 30 liver cells, sometimes centrally placed, but more often in the middle of the lobule part way between a portal space and a central vein. In these zones the liver cells were extremely vacuolated with no evidence of normal granulation and the nuclei showed extensive fragmentation. Frozen sections stained with Sudan III showed these cells loaded with fat. Leucocytes and highly phagocytic clasmatocytes infiltrated these areas, with destruction so great that the vessels could not be analyzed. There were also degenerations in the convoluted tubules of the kidney, some of which contained cellular debris and some fragments of red cells. Thus the effects of the protein in this animal were a specific leucocytosis, fever, an increase in clasmatocytes in the lung, multiple hemorrhages, focal fatty necrosis of the liver cells, and some damage to the cells of the kidney tubules.

TABLE I.

Protocols.

No. of rabbit	Dose	Length of life
	All protein fractions given intravenously	
R 138	1 dose of 10 cc. of 903	Died immediately after first dose
R 132	2 doses of 10 cc. of 903	Died after second dose
R 152	2 doses of 20 cc. of 304	Killed 48 hrs. after second dose. Maximum temperature 107.6°
R 131	26 doses of 2 to 5 cc. of 903. Total of 72 cc. in 33 days	Killed 3 days after last injection. Maximum temperature 107°
R 137	25 doses of 1 to 3 cc. of 903. Total of 32 cc. in 31 days	Died immediately after last injection; complicating peritonitis found, due to *B. lepisepticum.* Maximum temperature 108.2°
R 129	13 doses of 6 to 10 cc. of 304. Total of 126 cc. in 15 days	Killed 24 hrs. after last dose. Maximum temperature 108.5°
R 130	12 doses of 6 to 10 cc. of 304. Total of 116 cc. in 15 days	Died night after last dose. Maximum temperature 108.3°
	Phosphatide fractions	
R 156	2 doses of 80 mg. of A-3 intravenously	Acute death suddenly after second dose, from emboli
R 153	2 intravenous and 12 intraperitoneal doses of from 80 to 160 mg. of A-3. Total of 1280 mg. in 15 days	Killed 24 hrs. after last dose
R 160	12 intraperitoneal doses of from 80 to 160 mg. of A-3. Total of 1120 mg. in 13 days	Killed 48 hrs. after the last dose
R 158	1 intravenous and 12 intraperitoneal doses of from 40 to 160 mg. of A-4. Total of 1000 mg. in 14 days	Killed 6 days after the last dose
R 159	1 intravenous and 12 intraperitoneal doses of from 40 to 160 mg. of A-4. Total of 1120 mg. in 14 days	Killed 4 days after the last dose
	Controls	
R 243	15 intraperitoneal doses of from 80 to 122 mg. of lecithin. Total of 1242 mg. in 17 days	Killed in excellent condition 24 hrs. following the last injection
R 244	15 intraperitoneal doses of from 80 to 122 mg. of lecithin. Total of 1242 mg. in 17 days	Killed in excellent condition 24 hrs. following the last injection

TABLE I—*Concluded.*

No. of rabbit	Dose	Length of life
	Controls—*Concluded*	
R 245	15 intraperitoneal doses of lecithin plus tubercle bacilli, H 37, inactivated at 60° for 1 hr. Total of 1242 mg. lecithin and 3.75 mg. bacilli in 17 days	Killed in excellent condition 24 hrs. following last dose
R 246	15 intraperitoneal doses of lecithin plus tubercle bacilli, H 37, inactivated at 60° for 1 hr. Total of 1242 mg. lecithin and 4.00 mg. bacilli in 17 days	Killed in excellent condition 48 hrs. following last injection
R 247	12 intraperitoneal doses of tubercle bacilli, H 37, inactivated at 60° for 1 hr. Total of 3 mg. in 14 days	Killed in excellent condition immediately after last injection
R 248	16 intraperitoneal doses of from ¼ to 3 mg. of tubercle bacilli, H 37, inactivated at 60° for 1 hr. Total of 7 mg. in 19 days	Killed in excellent condition 24 hrs. after last injection

With the studies on toxicity just cited as a basis for dosage the remaining material was given to a small number of animals, rather than less amounts to a larger series in the hope of securing if possible a striking and characteristic effect. The results have seemed to justify this plan. Two rabbits (R 131 and R 137) received the protein 903 in repeated daily doses of 1 to 2 cc. of a saturated solution, with final doses of from 3 to 5 cc.; while two others (R 129, R 130) were given daily intravenous injections of 304 in the original amounts of 10 cc. per dose. With these dosages in total amounts as shown in Table I, the effects noted in the four animals were in general comparable.

Chart 2 is given as representative of the two animals with 903, Chart 3 for the two with 304. These charts are given for the sake of comparison of the effects on the peripheral blood of the chemical fractions from tubercle bacilli with the known effects in the rabbit of the living tubercle bacilli in large doses. In an earlier paper in this *Journal* (11) it has been shown that the changes in the blood after an intravenous injection of 1 or 2 mg. of bovine tubercle bacilli reflect directly, and may be interpreted in terms of, two different processes. First, an anemia combined with the fall in platelets and granulocytes indicates a direct effect on the bone marrow; second, an increase in monocytes and at times in clasmatocytes combined with a fall in lymphocytes is correlated with the general progress of the tubercular process in the tissues (6).

It has been demonstrated that the effect revealed in the blood in red cells, plate-

lets, and granulocytes is due to an extensive involvement of the marrow with tuberculosis, a constant finding at a certain stage with the doses mentioned; that the local tubercular lesions are sufficient to displace the fat and to reduce the marrow to the level of the early erythroblasts for the red cells and the early myelocytes for the white cells. Rabbit R 131 received 903, in doses as seen in Chart 2. It will be seen in this chart that there is a sharp fall of the red cells, correlated with a drop in hemoglobin beginning after the 8th dose of protein; the lowest point was reached Dec. 30 with 3,760,000 red cells. There was later a partial recovery of the red cells, but to an average of 5,000,000 rather than to the original average of 6,000,000. This fall in red cells was not correlated with a fall in granulocytes,

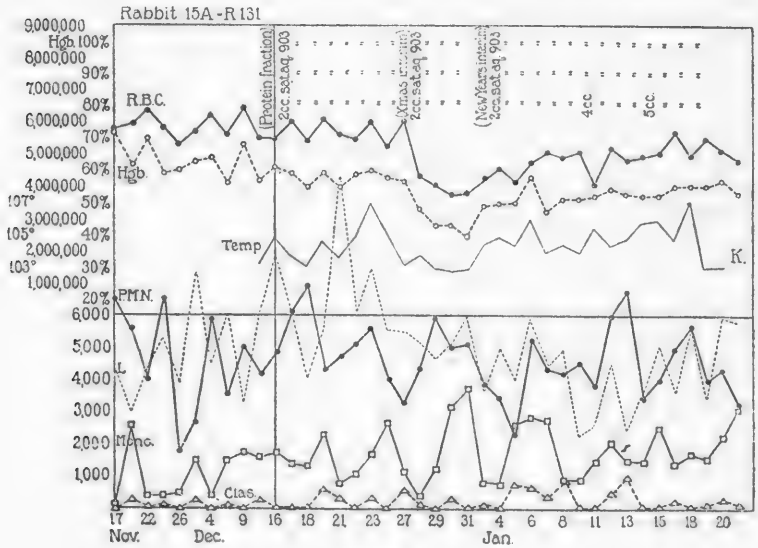

CHART 2.

the latter remaining practicly at their original level. The anemia was found to be due to multiple hemorrhages and was not therefore a local effect on bone marrow except in so far as there were, as in every case, hemorrhages in the bone marrow itself as well as multiple ones outside the marrow. The anemia was thus secondary to the hemorrhage.

In regard to the white cells, the total numbers are not shown on the chart but their range before the injections was from 7250 to 15,600; while after the injections there were four moderately high counts: Dec. 21, 17,000; Dec. 23, 15,600; Dec. 31, 15,400; and Jan, 6, 15,900. These numbers are not excessive for the rabbit and the experiments already recorded with R 132 and R 152 indicate that a marked

effect of the protein on the leucocytes is obtained only with larger doses. The chart also differs from a typical tubercular chart in a negative effect on lymphocytes. On the other hand, there was a somewhat rhythmic rise in monocytes and clasmatocytes which is like tuberculosis. At the times of the increase in monocytes in the peripheral blood there were some qualitative changes, namely some decreased motility, an increase in small scattered neutral red bodies in the cells, and some cells with accentuated rosettes; but no true epithelioid cells were found such as had been seen in the preliminary tests with 304 (6).

Rabbit R 137 received smaller doses of 903, namely, twenty of 1 cc., three of 2 cc., and two of 3 cc., making a total of 32 cc. as contrasted with a total of 72 cc. for R 131. R 137 had the only complication of the series, namely a terminal peritonitis due to the *B. lepisepticum*, identified by Dr. Ida Pritchett. The only difference between this chart and that of R 131, as far as the white cells were concerned, was a fall in both leucocytes and lymphocytes during the last 4 days. In this animal there was no anemia. The autopsy showed only small fresh hemorrhages so the interpretation is that the animal did not survive the hemorrhages long enough to show their effect in the peripheral blood. The curves of monocytes and clasmatocytes were the same as in the corresponding rabbit, R 131.

The peripheral blood in the two animals, R 129 and R 130 (Chart 3), that received the protein fraction 304, showed the same effects as 903. R 129 received eleven doses of 10 cc. and one of 6 cc. Rabbit R 130 had ten doses of 10 cc. and one of 6 cc. Both animals showed a gradually progressive anemia; the lowest count on R 129 was 3,680,000 from an original base line of 5,500,000; while the lowest count on R 130 was 2,970,000 from a level of 5,000,000 to 6,000,000 before the injections. Of all the animals with the protein fractions, R 130 showed the most marked anemia and had the most extensive hemorrhages. In both R 129 and R 130 there was a neutrophilia and a tendency toward lower lymphocytes. In R 130, though not in R 129, there was the same somewhat rhythmic rise in monocytes as is shown on Chart 2.

In summary,the protein fractions 903 and 304 were both toxic; both produced anemia with neutrophilia of greater or less degree; four of the rabbits died, two (R 138 and R 132) in the acute reaction to large doses of 903, one (R 130) after repeated doses of 304, and the other (R 137) after 903 but with a complicating infection so that death could not be ascribed to the protein alone. There were no signs of secondary infection in the other animals. One rabbit (R 152) was killed when in a critical condition; the other two (R 129, R 131) were killed while still in good condition All the rabbits appeared more or less ill with temperatures reaching between 107° and 108.5°.

At autopsy the findings in the series were quite uniform. The only signs of abnormality obvious in the gross inspection were multiple hemorrhages. In every animal, on opening the abdominal cavity, there was found edema of the prevertebral connective tissues, with small hemorrhagic foci scattered throughout from diaphragm to pelvis. These hemorrhages extended characteristically into the septa of the psoas muscles on either side and in one animal were found to have

dissected into the prevertebral tissues of the thoracic cavity and were found also in the axillæ. The question arose as to whether the psoas hemorrhages, so uniform in distribution in the gross, could be traumatic in origin, but they were present in only these five out of more than seventy rabbits handled in the same way by the same people; moreover, all the rabbits in this group had also hemorrhages in the bone marrow as well as in some of the other organs. The animal with the infection (R 137) had a peritonitis with a thick, purulent exudate covering the entire peritoneal surfaces, but typical hemorrhages were found in sections of the subperitoneal prevertebral tissues.

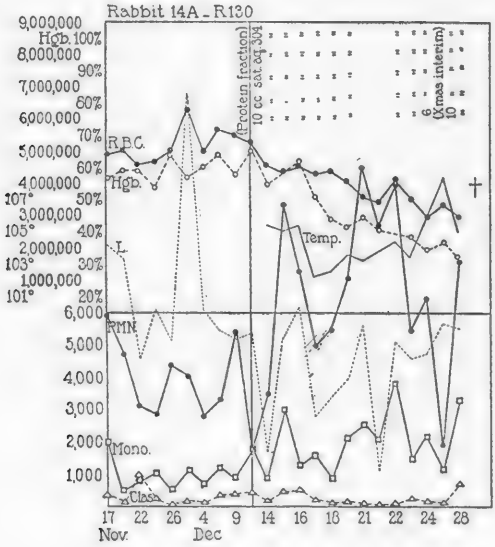

CHART 3.

In Fig. 6, is shown a drawing from the central part of the mesenteric lymph gland of Rabbit R 131, which drained a hemorrhagic area. The lymph cords are covered, or, in other words, the lymphatic sinuses are lined by endothelial cells which are engorged with debris from red cells. In this lymph gland the peripheral sinuses were entirely denuded of endothelium leaving quite bare the scanty framework of reticular cells which showed no phagocytic activity whatever. All the central sinuses of the gland show a complete phagocytic endothelium except for an occasional endothelial cell which has dropped off as is indicated by the arrow *b* (Fig. 6). At the point marked with the arrow *a* on the drawing there is a reduplication of the endothelium, with the outer cell rounding up as if to des-

quamate. At the point marked *c*, there is an endothelial cell stretching along the reticular framework. Within the lymph cords there are a few phagocytic cells exactly like the lymphatic endothelium; some of these are obviously adventitial cells to the blood vessels of the lymph cords; others appear to be free of any relation to the adventitia of a blood vessel. There was no involvement of the blood vascular endothelium *per se* in the phagocytic activity. In some of the central sinuses there were great numbers of free round phagocytes loaded with the debris, which cells probably account for the endothelium of the denuded peripheral sinuses. This lymph gland offers as good evidence as can be had that certainly some of the cells of the free phagocytic type, the typical clasmatocyte, may originate from endothelium, in this case from lymphatic endothelium. It also gives quite conclusive evidence that there are two types of cells in the lymphatic sinuses, the nonphagocytic reticulum and the potentially active endothelium; and it shows as well that the endothelium is complete or incomplete according to the functional state of the gland. It is probably true that the extensive response of the endothelium in this lymph gland was not a primary effect of the protein injected but was rather a response secondary to the presence of a massive amount of the debris of red cells. In this rabbit there was one rather large hemorrhage in the edge of the psoas muscle near the kidney and there are clumps of clasmatocytes loaded with yellow debris and others with brown pigment from the red cells in the surrounding tissues. This is the only rabbit in the series in which there has been any breaking down of the hemoglobin in any of the hemorrhagic areas sectioned. On Chart 2 from this animal (R 131), it will be seen that there was a sudden marked fall in the red cells, 24 days before the date of the autopsy; whereas on Chart 3, the fall in the red cells was a very gradual one. All the other hemorrhagic areas studied in the other animals show the condition illustrated in Fig. 9, in which there are intact free red cells in the tissues exactly like those in the vessels without any appreciable phagocytosis. Obviously there has been a marked increase in connective tissue cells, with many mitoses present. In the supravital studies of the tissues adjacent to that of Fig. 9, the predominating cell was identified as the clasmatocyte in its characteristic reaction to neutral red but no phagocytosis of the red cells was found. The interpretation is that these areas show a preliminary increase in the clasmatocytes by division before the red cells are engorged. In the fixed tissue, as shown in Fig. 9, it is clearly not possible to discriminate between fibroblasts and clasmatocytes; and it is well known that the inactive clasmatocyte has never been discriminated in fixed tissues. With the supravital technic as applied to the connective tissues, however, it is possible to distinguish readily the cell types morphologically differentiated and known as the fibroblast, the resting clasmatocyte, the stimulated clasmatocyte, the epithelioid cell, and the serosal lining cell (see plates, etc., in preceding papers (4, 6)).

Beside the hemorrhages, all the rabbits with the protein fractions showed a thickening of the interalveolar septa in the lungs. The maximum grade of this lesion found in any of the series is shown in Fig. 7. In the gross specimens the lungs did not look abnormal, but there was an increased resistance to the knife on cutting

and the supravital preparations all showed an increase in free cells which were of the clasmatocytic type. In all the lungs the areas of thickened septa were in patches and varied in amount from a lesion just perceptible up to the grade shown in Fig. 7. This section is from a rabbit, R 130, which was found dead. The lungs were mottled, which was postmortem, but supravital preparations were recorded as showing a marked increase in free cells, entirely due to clasmatocytes which were still living as shown in their characteristic reaction to neutral red. It is interesting to note that this type of reaction has already been found in response to the injection of both living and dead tubercle bacilli. It is this reaction which Lewis and Sanderson (30) found in the lungs 48 hours after the injection of massive doses of living bacilli, demonstrated to be an increase in clasmatocytes in another paper in this *Journal* (3). The reaction in the lung to the injection of dead bacilli or of chloroform extracts has been described in the literature (14, 18, 31) as an interstitial pneumonia, which is the lesion illustrated in Fig. 7.

In describing the autopsy results in Rabbit R 152, with massive doses of 304, it was noted that there were small necrotic areas in the liver. There was another rabbit in the series, R 130, with extensive acute necrosis of the liver cells. At autopsy, the hepatic lesions were easily visible in the gross, giving a marked mottling to the surface; frozen sections showed areas of cells loaded with fat and in fixed sections these zones showed extreme necrosis of the liver cells with destruction of their nuclei. The lesions are the same as in Rabbit R 152 but much more extensive. These are acute lesions, having no relation to the chronic periportal cirrhosis found in all the rabbits of the series. Inasmuch as the acute necrosis of liver cells was obvious in only two of the five animals, it cannot be regarded as a constant effect of the protein; it must, however, be kept in mind in the further biological testing of the material.

The lesions found in all the animals that received the protein fractions were multiple hemorrhages from small vessels and an increase in clasmatocytes, especially in the so called interstitial pneumonia of the lungs. These proteins were thus toxic to endothelium. In general, the proteins were toxic to the animal, lethal in large doses, and gave high temperatures.

Phosphatide Fractions.

Known weights of the phosphatide fractions were rubbed into a fine emulsion in water freshly distilled from glass. The dose determined with reference to the proportion of this material to the total weight of the dry bacilli was 80 mg. The first injections were made intravenously but since one of the animals dropped dead instantly, probably from an embolus, the intraperitoneal route was substituted.

Four rabbits were given repeated intraperitoneal doses of the two fractions, as shown in Charts 4 and 5. Two rabbits, R 153 and R 160, received A-3, while two rabbits, R 158 and R 159, had A-4. These two fractions were entirely nontoxic and there was no rise in temperature in any animal. The two rabbits that received A-3, of which Chart 4 is representative, both showed an anemia with a

fall of about 2 million red cells and a corresponding drop in the hemoglobin. At the same time both of them showed a rise in monocytes reaching the level of 4000 cells per c.mm. The two rabbits that received the fraction A-4, of which Chart 5 is representative, showed the same response in less degree. In these animals, R 158 and R 159, the fall in red corpuscles was a little over a million cells while the rise in monocytes was to the level of 2000 cells. The changes in leucocytes and lymphocytes were inconstant and perhaps negligible; Rabbits R 153 (A-3) and R 159 (A-4) both showed the slight drop in leucocytes and lymphocytes indicated on Chart 4, while the other two had no changes, as shown on Chart 5. Thus all

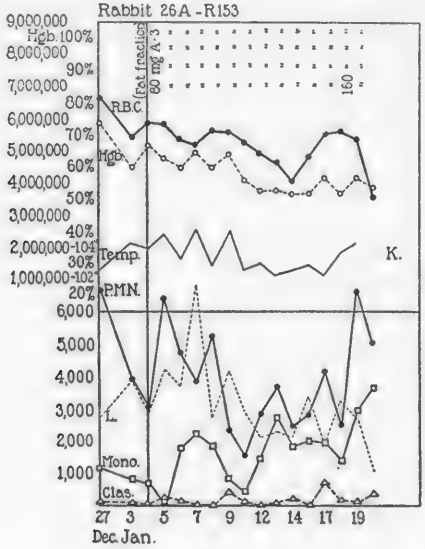

CHART 4.

four animals showed some anemia, which was possibly due to the distilled water introduced, since there were no hemorrhages and no changes were detected in the bone marrow. In all four animals there was a rise in monocytes, after A-4 to 2000 cells, after A-3 to 4000 cells. Thus the greater anemia and the more marked monocytosis were with the fraction A-3.

The four animals with the phosphatide fractions, in striking contrast to those receiving the protein, showed no clinical symptoms whatever during the course of the injections. The rabbits, therefore, were autopsied while all were yet in excellent condition when all the available material had been used. The extent of the pathological lesions was thus entirely unexpected. The findings in general

were identical in type in the four animals and the pathology in general was confined essentially to the peritoneal cavity. The local lesions were in every case so massive that it was difficult to differentiate quantitatively their relative extent in the individual cases. Rabbit R 128, which received A-4, had perhaps the greatest amount of involvement though it was one of the rabbits with the lesser reaction in the peripheral blood.

On opening the peritoneal cavity there were no ascites and no adhesions but all the animals showed an extensive involvement of the parietal and visceral peritoneum with what proved to be typical tubercular tissue. The omentum in every case

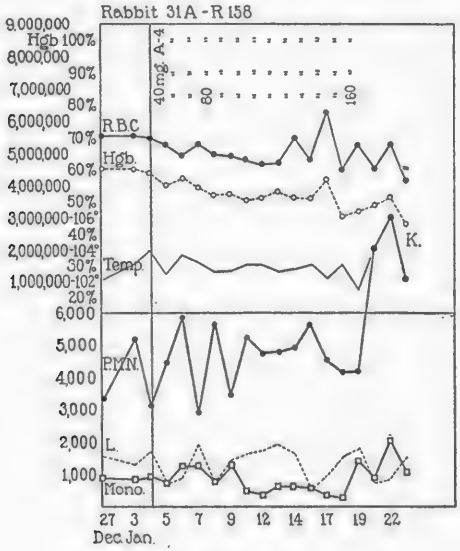

CHART 5.

was a dense thickened, nodular, dull reddish mass, markedly different from the delicate membrane of the normal state. The characteristic color was probably due to the increased vascularization. Some idea of the thickness of these omenta may be obtained from Fig. 11, in which the original thickness of the omentum illustrated can be seen at the upper right hand corner; the rest of the photograph gives about one-third of the actual thickness of the tissue at that point. The proliferation is specifically of epithelioid cells with a great excess of giant cells of the Langhans type in this area. The small darker nuclei indicate clusters of lymphocytes. Fig. 10 illustrates another area in the same animal (R 153) with fewer giant cells and a marked proliferation of typical epithelioid cells. The

predominating cell from the omentum of this animal is shown in the supravital technic as Fig. 5. This epithelioid cell is to be compared with a similar one, Fig. 3, which was found in the peripheral blood of a rabbit, R 19, 16 days after the injection of 1 mg. of living bovine tubercle bacilli. The graph showing the peripheral blood of this animal is given as Chart 1 in another paper in this *Journal* (11), and the cell was drawn on Dec. 2 when there were 6000 monocytes per c.mm. in the peripheral blood. In both of these epithelioid cells is shown the typical rosette of tiny bodies, with the carmine reaction to supravital neutral red, which is the characteristic of the cell that makes the tubercle. In contrast to these epithelioid cells (Figs. 3 and 5) which far outnumbered any other type of cell in the lesion, are three other cells illustrated from the same omentum as the cell of Fig. 5. Fig. 1 was an unstimulated fibroblast, entirely typical of the type. The cells of Figs. 2 and 4 were in our interpretation somewhat stimulated, rounded up clasmatocytes. They are to be compared with the unstimulated clasmatocyte of the omentum shown in Fig. 3 from an accompanying paper in this *Journal* (3). This cell was from the omentum of another of the rabbits, R 160, that received the phosphatide fraction A-3 , and was identical with many found in R 153. But beside these long forms there were also found cells, such as the ones shown in Figs. 2 and 4. In our interpretation these latter cells were types to be classified as clasmatocytes. The cell of Fig. 2 is the more typical; the cell of Fig. 4 showed the tendency of the smaller vacuoles to fading toward yellow; the vacuoles when originally seen were a deeper red than is shown in the drawing, but they were ultimately orange and yellow in color while all the epithelioid cells in the preparations still showed an unchanged carmine reaction. Reference to the photograph, Fig. 10 (R 153), from the connnective tissues near the bladder, shows the predominating cell to be the epithelioid type, with eccentric nucleus, but there is an occasional atypical cell with the nucleus in the center as in Figs. 2 and 4. Proof will be given in the discussion of the controls that the clasmatocyte as well as the monocyte takes in lipoids. However, the contrast in the reaction of the cells under discussion in an environment as identical as there would be within the area of omentum included in one oil immersion field of the microscope is strikingly shown in Figs. 2, 4, and 5, or in Figs. 2 and 3 in the accompanying paper in this *Journal* (3).

These are the observations. Concerning them, two different interpretations are possible; either the same type of cell is responding in two different ways, thereby making it necessary to postulate differences in the environment; or, two potentially different strains of phagocytes are reacting differently in the same environment in their disposal of phagocytized material. Our interpretation at the moment favors the latter explanation as the more probable.

In all four of the phosphatide animals there was marked involvement of the parietal peritoneum, especially near the site of injections. The visceral peritoneum covering the pelvic organs and the mesentery were extensively involved while the surface of the intestine had everywhere patches of the same tubercular tissue that characterized the omentum. Most of the lesions were nodular, white

or of the same dull reddish color as the omentum. In Fig. 8 is shown a section of the duodenum at the site of involvement in Rabbit R 159. It shows that the lesions are here limited to the serosal coat. The magnification is low to show the extent of the new growth. The mass of the tissue is of epithelioid cells. The dark patches in the center are lymphocytes; the two dark areas on the left show necrosis with infiltration of leucocytes. The zone in the upper left corner is the more markedly necrotic. In one of the animals there was some involvement of the submucosa, probably by extension. In this same animal (R 159) there was an occasional small tubercle in the liver and a few epithelioid cells in the lung, but in general the reaction was almost entirely localized to the peritoneum. In one animal only, R 158, was there an involvement of the mesenteric lymph glands, as shown in Fig. 12. Here the peripheral follicles are seen extensively involved with typical epithelioid cells, giving a picture that would be described as early tuberculous involvement, though no acid-fast bacilli could be found.

Complete parallel surveys of all tissues from all animals receiving either the protein or phosphatide fractions have yielded uniformly negative results with the Ziehl-Neelsen technic. The phosphatide material itself gave a diffuse reddish reaction with the carbolfuchsin and some of the large giant cells, such as are shown in Fig. 11, stained a faint, diffuse pink. In the case of the lymph gland of Fig. 12 there were certain areas within the follicles and in the reticulum of the lymphatic sinuses in which groups of cells were found containing acid-fast debris. There were, however, no intact, acid-fast bacilli found in any instance.

The contrast between the reaction of the connective tissues to the protein fractions on the one hand and to the phosphatide material on the other may be seen at a glance in comparing the tissues of the lung, as shown in Fig. 7, with the omentum of Fig. 11. In Fig. 7, the reaction is not at all that of tuberculosis. The contrast is, however, much clearer in Figs. 9 and 10, where the tissues involved are entirely comparable. Fig. 9 is from the prevertebral tissues near the kidney after the protein, and Fig. 10 from the connective tissue near the bladder after the phosphatide fraction. The character of the round cells with eccentric nuclei, together with the typical giant cells of a tubercular lesion in the latter, contrast markedly with the long and the branched cells with no trace of a tubercular reaction in the former. In both of these photographs there is shown a little perivascular reaction. In none of the tissues in the entire series was any tendency towrad fibrosis seen. In the tubercular lesions after the phosphatide fractions there was a considerable new growth of vessels.

These are the preliminary tests of substances from the chemical analysis of the tubercle bacillus. Under the plans of the Research Committee of the National Tuberculosis Association, Dr. William Charles White, chairman, these tests are to be extended, both to the products from the further analysis of these protein and phosphatide fractions and to comparable substances from other organisms.

As far as these studies have progressed, it is clear that certain of the responses to the proteins are like those to other proteins; for example, the anaphylactoid response to large doses, and the direct effect on the neutrophilic leucocytes from an excessive dose, and possibly the temperature. The acute effect on the liver cells in two animals can only be evaluated with a larger series of animals. With the proteins, all the rabbits showed fever, multiple hemorrhages, and a tendency toward an increase in the cellular response of clasmatocytes. How far the toxic effect on endothelium and the pressor effect on clasmatocytes are specific awaits further tests. The phosphatide fractions showed only one response, namely the local production of the typical lesion of tuberculosis. There was a massive local increase in monocytes, epithelioid cells, and Langhans giant cells; there was some infiltration of this tubercular tissue with lymphocytes and there was some necrosis. Thus, with these two types of substances from the analysis of the bacilli many of the effects of the actual disease have been reproduced. The typical distribution of the lesions of the infection with living bacilli was not reproduced. If the observations that in the disease itself clasmatocytes fragment the living bacilli, that the clasmatocytes are in turn disintegrated, and the resulting debris rephagocytized, actually mean that large numbers of bacilli are killed and broken down by the cells of the animal, then the actual progress of a tubercular infection involves the effects of both living and dead bacilli on the body.

Controls.

Before venturing any interpretation as to the specificity of the above reactions of the fractions from tubercle bacilli, adequate controls are, of course, essential. It will be possible to secure soon various bacterial proteins with which to compare and contrast the reactions already noted with the protein fraction from the tubercle bacilli. In some respects the acute reactions with this material have approximated anaphylactoid phenomena; in other respects the responses have been unlike the non-specific protein reactions with which the literature deals. Therefore, the real significance of these observations awaits further studies.

As a partial control for the findings with the specific phospholipins of the tubercle bacilli, certain appropriate, if inadequate, experiments were carried out. When stained by the Ziehl-Neelsen technic, it was found that this material, as received, contained a limited number of intact, acid-fast bacilli, the protein fractions being free of such findings. However, when the protein and phosphatide fractions were injected into guinea pigs directly, or inoculated on Petroff's media,

or when the tissue suspensions from rabbits, treated as described above with these materials, were inoculated into guinea pigs, there was no evidence either from repeated tuberculin tests or at autopsy, that any of the bacilli were viable. Ten guinea pigs were used; five were autopsied 2 months after inoculation with completely negative findings, and five are still living and in perfect health 7 months after. Therefore, the control series of rabbits was divided into three groups: one to receive lecithin (12)[1] alone in doses comparable to that given in the case of the phospholipin; another to receive the same dosage of lecithin plus a known added weight of dead human tubercle bacilli, Strain H 37; the third to receive dead bacilli alone, all conditions being identical with those existing during the phosphatide series.

It has long been known that the injection of dead tubercle bacilli can produce lesions when injected into the living animal (13, 14). Koch (15) himself found that they caused aseptic pus upon subcutaneous inoculation. That the lesions produced with the dead organisms may be tubercles with typical epithelioid and giant cells is also well recognized (14, 16–20). Typical of this early work is that of Prudden and Hodenpyl (21) who in 1891 injected rather large amounts of dead bacilli, 2 to 3 cc. of a milky suspension, by various routes, subcutaneous, intrapleural, intraperitoneal, and intravenous. With the intrapleural and the intraperitoneal routes, two animals, one in each series, developed a few small nodules of epithelioid cells in the serosa. With the intravenous route, the tubercular tissue was confined to the lungs. Prudden (22) then studied the lungs after intratracheal injections, in which he also found some reaction in the pulmonary tissues.

In the attempts to explain the mechanism of the reactions noted, the nonspecific foreign body stimulus of the bacteria as such is mentioned (18, 23), but it is evident that the preponderant opinion of investigators has been that the histological lesion caused by the tubercle bacillus is due to a poison liberated from the body of the bacillus by the action of the tissue cells (13, 14, 17, 23, 24, 25). Among the first to attempt an analysis of the effects of chemically separate constituents of the dead tubercle bacilli was Weyl (26). He obtained two substances from the bacillus, one of indifferent nature with the tinctorial properties of the bacteria, and the other a toxomucin which, upon subcutaneous injection, caused dry necrosis of the skin. Auclair (27) reported the production of caseation necrosis in guinea pigs by subcutaneous and intratracheal injections of lipoidal materials from human tubercle bacilli extracted by ether, chloroform, xylene, and benzene. In 1903 he studied the tissue reactions following ether extracts of typhoid, Friedländer's, and Loeffler's bacilli, streptococcus, *Staphylococcus aureus*, gonococcus, and actinomyces, finding in contrast to the effects of extracts of tubercle bacilli no typical epithelioid proliferations but rather in each instance only inflammatory lesions comparable to those resulting from the original organism in its usual manifestations. Morse and Stott (23) produced typical cellular tubercles, without caseation, in rabbits and white rats, by subcutaneous intraperitoneal, and intravenous injections of the alcohol extract, but found the

[1] We are indebted to Dr. P. A. Levene for the lecithin used in the control animals.

ether extract to be inert. They attributed the characteristic microscopic lesion to waxy substances acting as a peculiar type of foreign body. Gaehlinger and Tilmant (28) found non-specific "liver lipoids," when injected subcutaneously, capable of producing tubercles with caseation, though Ray and Shipman (29) could not confirm this observation. The latter investigators found chloroform-soluble lipins extracted from tubercle, grass, and colon bacilli to produce "entirely similar epithelioid tubercles" when injected *via* the subcutaneous and intrapulmonary routes in guinea pigs. The differences in the degree of the involvement were not considered significant, the conclusion being drawn that the characteristic histological features of tubercles are "merely a foreign body reaction." However, their controls of sodium stearate emulsion, with and without 0.5 per cent chloroform, and olive oil, with and without 0.5 per cent chloroform, gave no lesions. True caseation was never seen by them and they question previous findings of this nature because of the close macroscopic resemblance of the solid pus of rabbit and guinea pig to caseous material. Thus it will be seen that the literature provides to some degree controls for the work presented in this investigation. However, the chemistry of bacteria is being placed upon a new foundation today with the improved methods of quantity production of known strains of organisms on synthetic media of known composition, and with the development of chemical procedures yielding material in amounts sufficient both for analysis chemically and biologically. Also the newer criteria for cell identifications are making the study of the biological reactions more specific and exact than was possible in the earlier investigations.

Charts 6, 7, and 8 are representative of the peripheral blood, respectively, in the three control groups here cited. No changes whatever are revealed in any of the graphs representative of temperature, weight, red cells, hemoglobin, or white cells of the various strains, in the periods of experimentation following the control periods. The fluctuations noted are quite within the range of normal for each determination. Thus, the clinical and peripheral blood findings indicate even less of a disturbance in the normal mechanisms in these control animals than was the case with the phosphatide fractions from the tubercle bacilli.

Because of the intraperitoneal route for the injections and the striking findings with the phosphatide fractions limited essentially to the local manifestations in the peritoneal cavity, the greatest interest attaches to the survey of this region, particularly the peritoneum and omentum. Both parietal and visceral peritoneum in five of the animals were glistening, smooth, and uninvolved throughout; only in Rabbit R 245, of the second group, were there found several strong, fibrous adhesions extending between the site of injection in the left abdominal wall and the serosa of the descending colon; the latter were probably secondary to some trauma incident to one of the injections.

In the two animals receiving lecithin alone (R 243, R 244), the omentum in the gross showed a patchy, dusky, red color, with an increase in milk bodies, but lacked the extensive nodular thickening present in the animals receiving the phosphatide fractions. Supravital studies of films of omentum spread on neutral

red-Janus green slides showed a dilatation of the omental capillary bed accounting for the gross appearance. The reaction of the adventitial cells along the small veins was particularly prominent. The milk spots were focal accumulations of highly phagocytic clasmatocytes, their vacuoles reacting brilliantly with the neutral red. The phagocytized debris was globular but clearly not red cells. No monocytes or epithelioid cells could be identified as such. These identical preparations were subsequently fixed *in situ* in formalin, which faded the neutral red of the clasmatocytes, and were then subjected to staining with Sudan III. On reexamination of the same areas previously studied in the supravital, it was

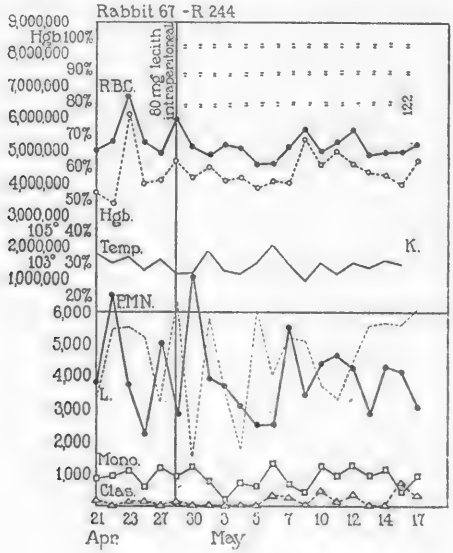

CHART 6.

found that the vacuoles formerly reacting to the neutral red also reacted to the fat stain, quantitatively, indicating a specific response to the foreign phospholipin of the strain of cells of the connective tissues chiefly responsible for the handling of non-specific debris.

The human tubercle bacilli, Strain H 37, used in the last four animals of this control group, were grown under the same conditions as those used for the chemical analyses yielding the proteins and phosphatides of this experiment, and were, indeed, dried weights from identical lots used by Dr. Johnson and Dr. Anderson. Known weights were inactivated at 60° for 1 hour and the individual dosage in all four animals was the same though the total dosage varied as indicated in Table I.

It was the desire to compare the relative reactions of known weights of dead bacilli alone *via* the peritoneal route with comparable dosages in the presence of a non-specific phospholipin.

The two animals receiving lecithin in dosages comparable to those of the phosphatide fractions, plus known quantities of inactivated human tubercle bacilli (R 245, R 246), also showed the evidence of dilated vessels and increased milk bodies in the omentum. However, supravital studies of omental spreads from these rabbits showed the predominating reaction in R 245 to be the typical epithelioid cells, with clasmatocytes, as in the preceding group, stimulated though

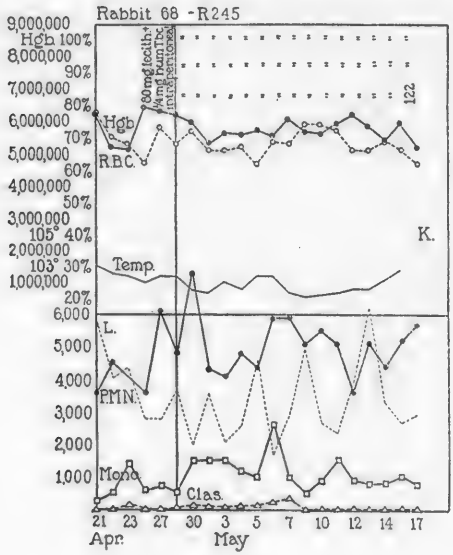

CHART 7.

less in number; while in R 246, the predominant reaction was clasmatocytic with a less pronounced response of epithelioid and giant cells.

In the two animals receiving dead bacilli only (R 247, R 248), the omentum had the gross appearance already noted. Supravital studies showed the more densely cellular areas to be predominantly clasmatocytic, though without the excessive phagocytosis of fat globules so prominently a part of the picture seen in the preceding four rabbits. The Sudan III preparations showed many of the cells characteristic of the clasmatocyte group with fine orange globules and an occasional larger fat body, but the majority of the cells did not react. This was in particularly striking contrast quantitatively to the studies in rabbits receiving the

lecithin. Typical epithelioid cells and giant cells of the Langhans type while present were a minority finding in these omenta subjected to dead bacilli.

Thus it will be seen that the intraperitoneal injections *per se* caused more or less of a vascular dilatation of the omentum, giving rise to a characteristic dull pink coloration, with a local cellular stimulation predominantly clasmatocytic in all the animals with the exception of R 245. Those rabbits receiving only lecithin showed no reaction of the monocytic strain of cells which could be identified with the usual criteria of the supravital technic, but rather there was a profound phagocytic response on the part of clasmatocytes as shown by surveys with

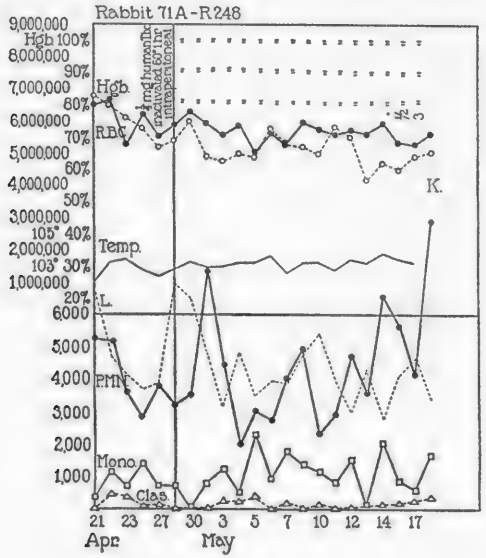

CHART 8.

Sudan III. The response of epithelioid cells was minimal except in the case of Rabbit R 245, in which both lecithin and bacilli had been given, and here there was nothing comparable in the extent and specificity of the reaction to that seen in any of the animals receiving the special phosphatide fractions from the tubercle bacilli.

These reactions of peritoneum and omentum are an adequate index of the general findings in the other tissues of the several groups. The mesentery showed some increase of cellular bodies comparable histologically to those described in the omentum. The mesenteric lymph glands in all animals were swollen, tense, and on section a copious flow of opaque fluid was released, which coagulated quickly

on standing. There was in every instance a great excess, four to five per oil immersion field, of typical clasmatocytes loaded with debris, but in all supravital studies only two typical epithelioid cells were seen (R 245) and no giant cells. The bone marrows in every animal were normal in their hemopoietic activity, and no epithelioid or giant cells were found in any case. There was only one instance in the control group of six animals in which there was an acute splenic tumor. The spleen of R 244 weighed 2.4 gm., but on supravital study showed only the normal cellular differential except for numerous red blood cells. Only one spleen (R 245) showed epithelioid cells, three in number.

Pleural cavity, lungs and heart, liver, intestinal tract, kidneys, from the animals receiving lecithin were negative in the gross and microscopically.

Both animals receiving lecithin plus bacilli showed some increase of monocytes in the lung with occasional typical epithelioid cells, but with the predominant cell being the clasmatocyte. The large intestine in R 245 was involved, possibly at the site of an injury incident to the injection, the small intestine being unaffected throughout.

Rabbit R 247, receiving dead bacilli only, showed a few epithelioid cells in the lung preparations, but in R 248 none were found, the usual clasmatocyte predominance being the obvious finding in both cases.

Certainly it may be accepted that dead bacilli alone in repeated doses and in the amounts cited, when injected intraperitoneally, can excite in the peritoneal cavity only a very limited development of the cellular pathology pathognomonic of tuberculosis; that lecithin is quite incapable of inducing other than a non-specific phagocytic response; and that the combination of lecithin and dead bacilli together reacts essentially independently with only a minimal response of epithelioid cells.

From the literature it is clear that the intravenous injection of dead tubercle bacilli produces in the lungs two distinct reactions: first, the so called interstitial pneumonia, primarily a proliferation of clasmatocytes; and second, small foci of typical epithelioid and giant cells. The protein and phosphatide fractions from tubercle bacilli here used sharply differentiate and separate these two reactions.

It may not be surprising to find the occasional epithelioid cell in other pathological cellular responses, for in the limited surveys with the supravital technic already made, they have been found in Hodgkin's disease and in the septa of certain tumors. The exact nature and distribution of the specific stimulant, possibly a lipoid, remain to be determined. However, the very special relationship

which this reaction bears to tubercular infection, and the apparent symbiosis of tubercle bacillus and epithelioid cell, present an approach to this disease which is thought at present to be significant.

In the further studies with the chemical products from the tubercle bacilli it will be important to analyze the biological reactions in terms of the phagocytic mononuclear cells of the connective tissues, with the possible differentiation functionally into monocytes, epithelioid cells, and clasmatocytes.

SUMMARY.

1. The clasmatocyte, the cell with the power of fragmenting tubercle bacilli, the cell making the lesion of the so called interstitial pneumonia, has been shown to be the overwhelming response to the special protein fractions, 304 and 903. Multiple hemorrhages, high fever, and toxicity have marked the use of these fractions in every instance.

2. The epithelioid and the giant cell of the Langhans type, making typical tubercular tissue, have been the massive and specific response of the peritoneal cavity to intraperitoneal injections of the phosphatide fractions, A-3 and A-4. These fractions have been entirely non-toxic in the dosages used.

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EXPLANATION OF PLATES.

PLATE 26.

FIG. 1. Typical resting fibroblast from the omentum of a rabbit, R 158, which had received 13 intraperitoneal doses of the phosphatide fraction A-4 on successive days, and was killed 6 days after the last injection. Stained in supravital neutral red. Magnification about 1940, as indicated by the red cell near Fig. 5.

FIG. 2. Stimulated, round clasmatocyte from the same preparation as the cell of Fig. 1.

FIG. 3. Epithelioid cell, stained supravitally with neutral red, taken from the peripheral blood of a rabbit, R 19, 16 days after the injection of 1 mg. of living bovine tubercle bacilli into the ear vein. The round, unstained bodies are fat. The chart of the blood of this animal is shown in another paper in this *Journal* (11) as Chart 5; there were 6000 monocytes in a c.mm. of the blood at the time, Dec. 2, when this cell was drawn. Magnification shown by the red cell.

FIG. 4. Slightly stimulated clasmatocyte from the same preparation as the cell of Fig. 1.

FIG. 5. Typical epithelioid cell, from the same preparation as the cell of Fig. 1, showing the rosette, with the carmine tone in supravital neutral red, characteristic of the type.

FIG. 6. Drawing of lymph cords and sinuses from the center of a mesenteric lymph gland of a rabbit, R 131, after 26 injections of the protein fraction 903. Tissue fixed in Zenker-formol. Stained with hematoxylin and eosin. Drawn with the camera lucida, magnification about 870. The lymphatic endothelium and the clasmatocytes which have become free from it are marked by their content of the debris of red cells from a hemorrhagic area which was being drained. *a*—reduplication of endothelium with the outer cell becoming rounded up; *b*—a denuded area where the endothelium has desquamated; *c*—an endothelial cell stretching across the sinus. In the center is a tiny lymph cord with a perivascular clasmatocyte; to the left are free clasmatocytes in the lymph cord.

PLATE 27.

FIG. 7. Photograph of a section of the lung from Rabbit R 130, which had received 12 intravenous injections of the protein fraction 304. Animal found dead the morning after the last dose. Shows the maximum thickening of the septa with clasmatocytes, the type proved by supravital studies since the cells were still living. All the tissues were fixed in Zenker-formol and stained in hematoxylin and eosin. × about 90.

FIG. 8. Photograph of the duodenum of a rabbit, R 159, which had received 13 intraperitoneal doses of the phosphatide fraction A-4, and was killed 4 days after the last dose. To show the extent of the serosal lesion. The mass of the tissue is of epithelioid cells, with the dark spots in the center of lymphocytes and two large masses of necrosis to the left. × about 9.

PLATE 28.

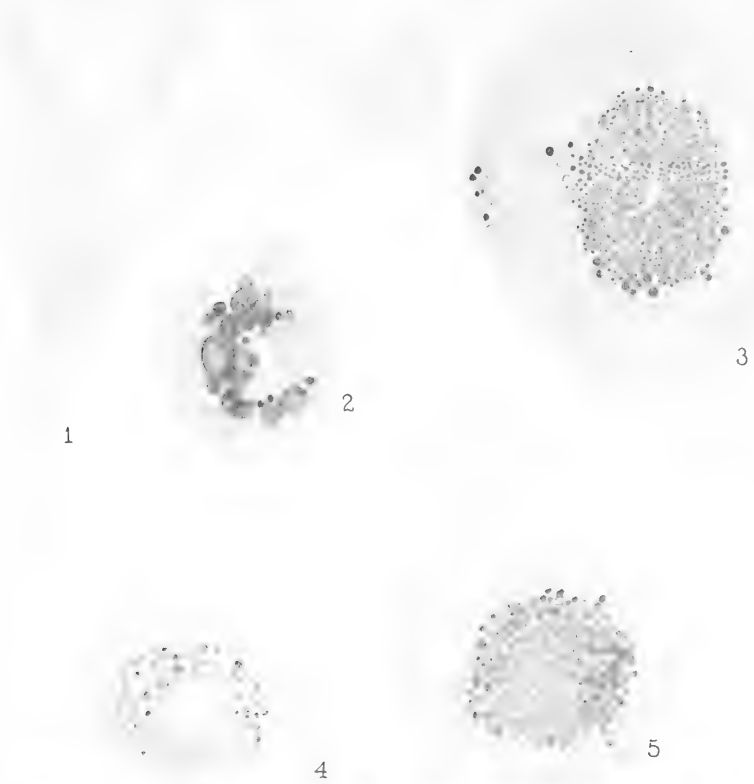
FIG. 9. Photograph of the retroperitoneal connective tissue, in the edge of the psoas muscle near one kidney, of a rabbit, R 129, which had 13 doses of the protein fraction 304. Killed 24 hours after the last dose. The tissue is hemorrhagic and edematous, and shows a stimulation of the connective tissue cells which were predominately clasmatocytic in the supravital studies. × about 455.

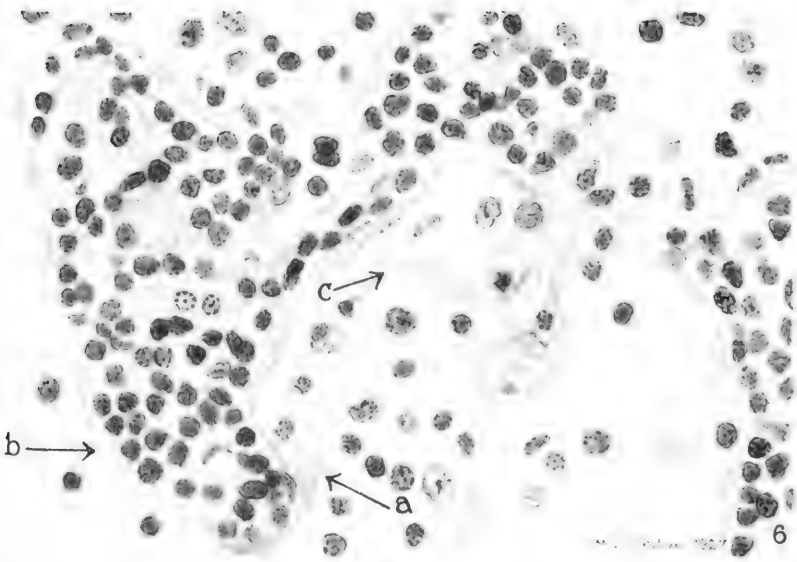
FIG. 10. Photograph of the connective tissue near the bladder from a rabbit, R 153, which received 14 intraperitoneal doses of the phosphatide fraction A-3, and was killed 24 hours after the last dose. It shows a reaction predominately of epithelioid cells and giant cells of the Langhans type. × about 455.

PLATE 29.

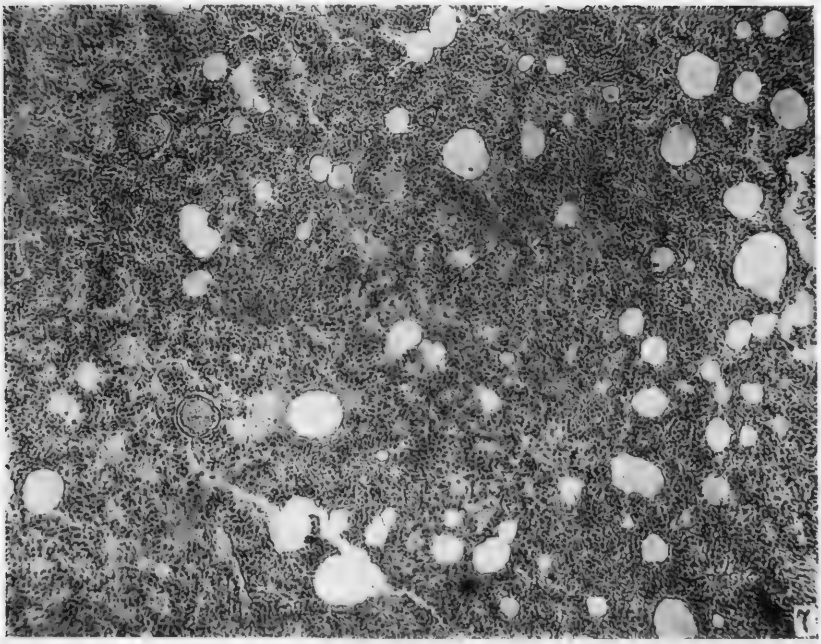
FIG. 11. Photograph of the omentum of a rabbit, R 153, which had received 14 intraperitoneal doses of the phosphatide fraction A-3, and was killed 24 hours after the last dose. It shows a reaction predominately of the giant cells of the Langhans type. There are many blood vessels and some clumps of lymphocytes. × 195.

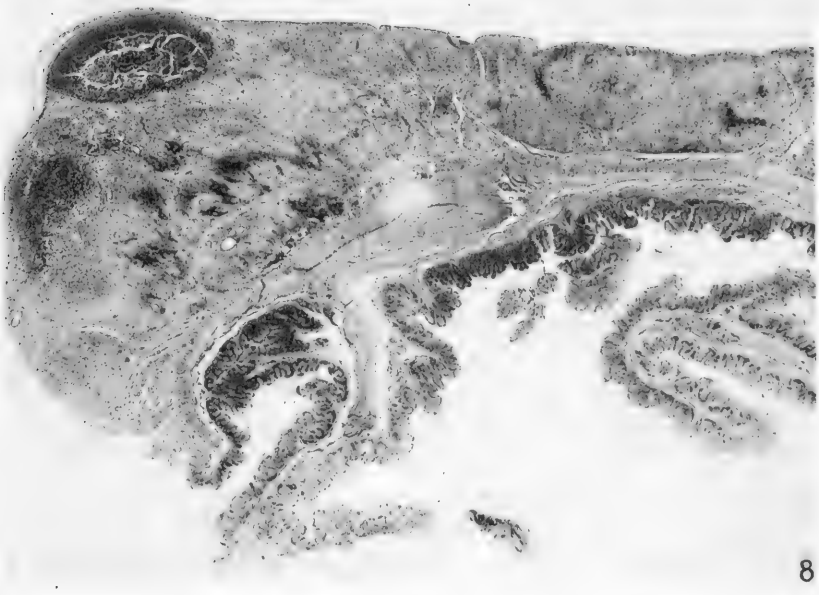
FIG. 12. Photograph of the mesenteric lymph gland of a rabbit, R 158, which had received 13 intraperitoneal doses of the phosphatide fraction A-4, and was killed 6 days after the last dose. The peripheral follicles with pale centers show extensive involvement with typical epithelioid cells. × about 90.





(Sabin and Doan: Chemical analysis of human tubercle bacilli.)





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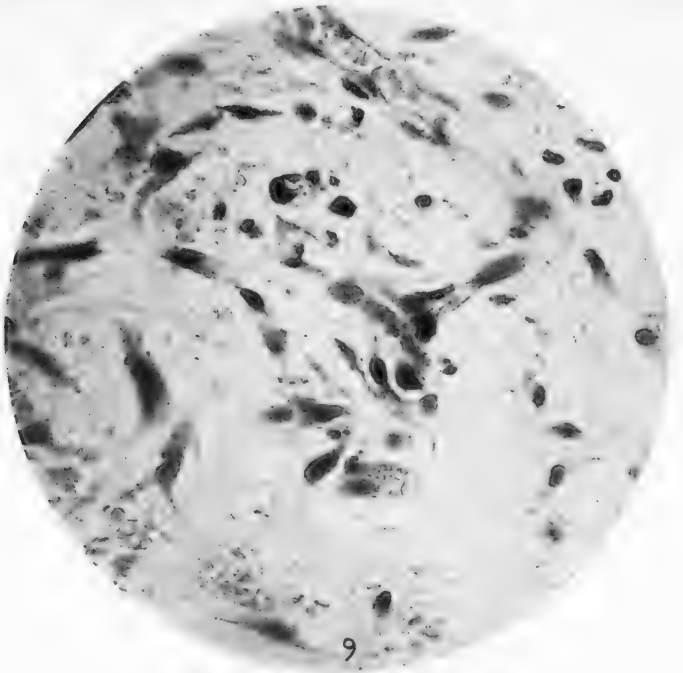
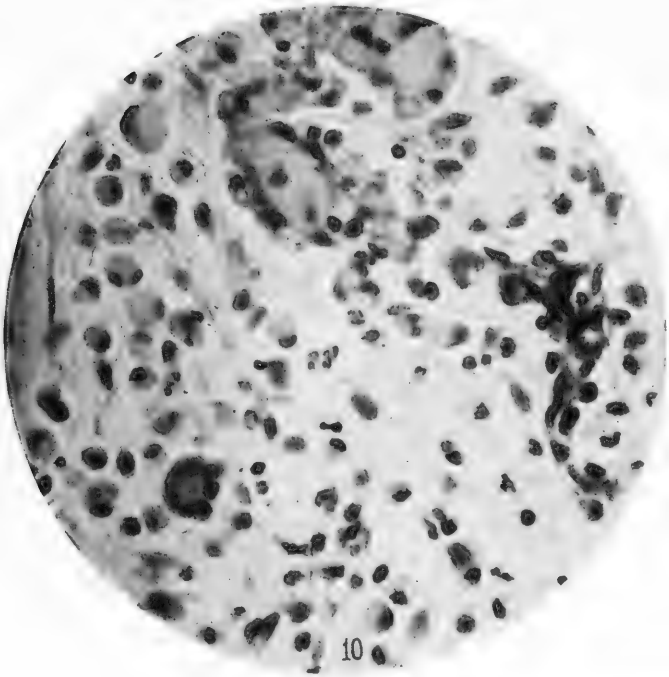
(Sabin and Doan: Chemical analysis of human tubercle bacilli.)





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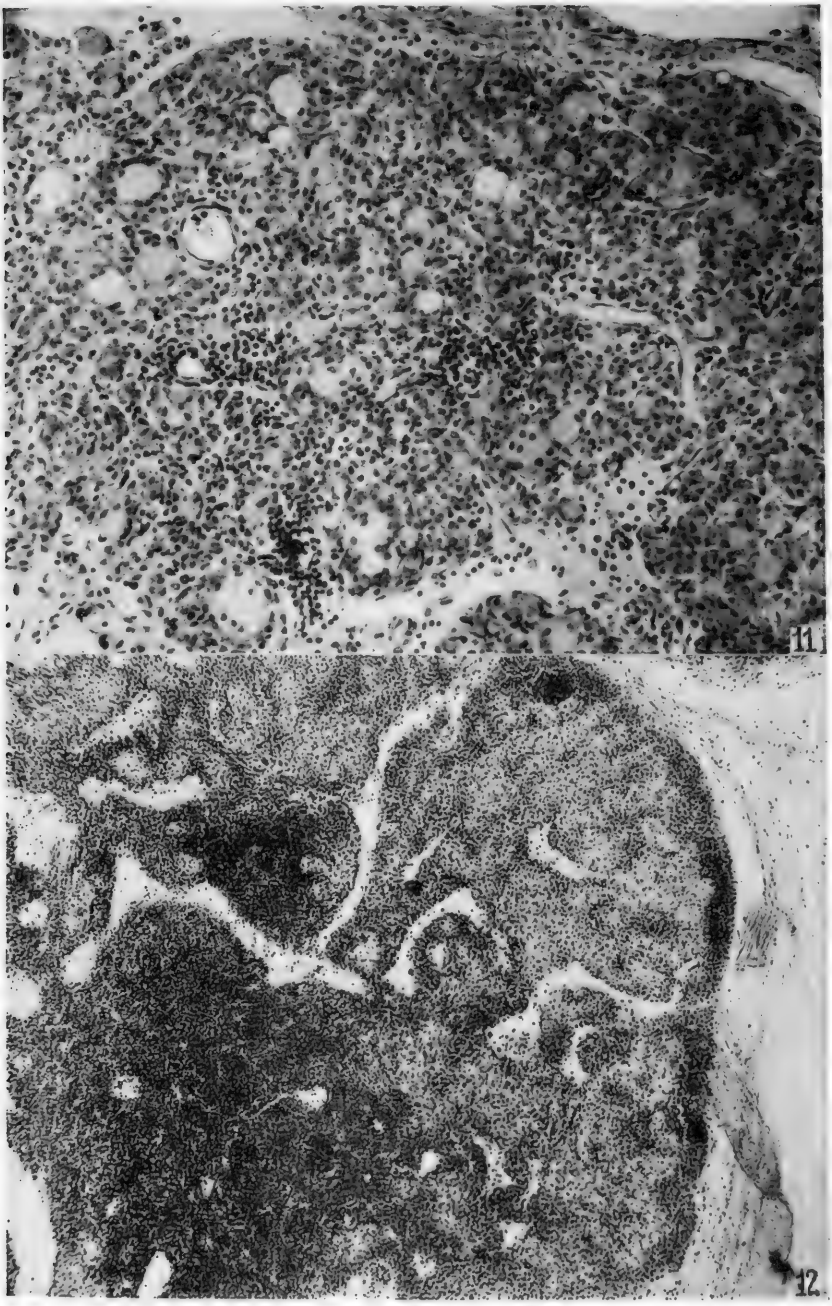

(Sabin and Doan: Chemical analysis of human tubercle bacilli.)

THE EFFECT OF SERUM UPON THE GERMICIDAL ACTION OF SOAPS.

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That serum inhibits the germicidal action of soaps was shown by Noguchi (1907) and by Landsteiner and Ehrlich (1908). Further observations were later made by Lamar (1911) and by Walker (1924), Walker being the first to show that the effect of serum upon soaps varied both with the soap used and with the organism.

In all these investigations, the effect of the pH was neglected. Serum that has come to equilibrium with the CO_2 of the air has a pH of 8.6 to 8.8. As the soaps having twelve or more carbon atoms are strongly hydrolyzed, their solutions are more or less alkaline. Hence, with no adjustment of the pH, the test fluid will have a reaction of pH 8.6 to 9.5 or more.[1] As a previous investigation (Eggerth, 1926) had shown that the germicidal action of soaps in salt solutions was greatly modified by the pH, it was determined to study the action of serum on soaps over a wide pH range.

EXPERIMENTAL.

Soaps of the following normal fatty acids were used: caprylic, capric, lauric, myristic, palmitic, and oleic. The soaps were prepared by adding the theoretical quantity of fatty acid (Eastman's) to N/5 KOH. Kahlbaum's sodium oleate was used. Just before each experiment, serial dilutions of the soap used were prepared in sterile N/10 NaCl solution.

The test organisms were: *Streptococcus pyogenes* ("Gay" strain); *B. diphtheriæ* ("Park-Williams No. 8"); *B. typhosus* ("Pfeiffer" strain); and *Staphylococcus aureus* (old laboratory culture). These are the same organisms that were used in the previous investigation (Eggerth, 1926).

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[1] The effect of the addition of boric acid to serum-soap mixtures, first described by von Liebermann and von Fenyvessy (1909), is due to the shift in the pH.

Fresh, sterile sheep serum was used. To every 10 cc. of serum, 1.0 cc. of N/5 HCl was added, to neutralize the $NaHCO_3$ present. The serum then stood overnight to allow the escape of the liberated CO_2; the pH was then about 7.2 to 7.6. It was then divided into five or more portions, and each portion adjusted with acid or alkali to the desired pH, the colorimetric method of Cullen (1922) being used to determine the end-point. The volumes of the different portions were then equalized with sterile N/10 NaCl. When it was desired to dilute the serum, this was done with sterile phosphate-glycine buffer mixtures of the corresponding pH. These buffers have a salt content of N/10; their composition is given in the first article of this series (Eggerth, 1926).

The serum mixtures were then inoculated with the test organism. The inocula

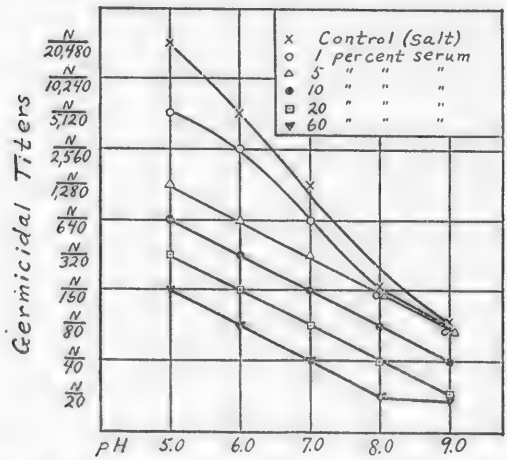

FIG. 1. The germicidal titers of laurate for *Staphylococcus*. The incubation period was 2 hours at 37°C.

were such that each 0.5 cc. of test fluid contained 0.04 cc. of broth culture of *Streptococcus pyogenes* or *B. diphtheriæ*, or 0.02 cc. of *Staphylococcus aureus* or *B. typhosus*. Then 0.5 cc. quantities were pipetted into series of small test-tubes. To each tube, 0.5 cc. quantities of soap dilutions were then added. (When a final serum concentration of 60 per cent was desired, 0.75 cc. of adjusted serum and 0.25 cc. of soap dilution were used.) Finally each tube was rotated while in a slanting position and placed in the water bath at 37°C. At the end of 30 minutes, 2 hours, and 18 hours, a 4 mm. loopful from each tube was subcultured on plates of blood agar (*Streptococcus* and *B. diphtheriæ*) or plain agar (*Staphylococcus* and *B. typhosus*). At the close of the experiment, the pH of each tube was tested with the appropriate indicator. When substances other than serum (egg white, gela-

tin, etc.) were tested, the same general procedure was followed. The final concentration of salt in the tubes was N/10 or a little greater.

The results of some typical experiments are shown in Figs. 1 to 3. In each case, the curve for a particular concentration of serum bears a definite relationship to the curve obtained for salt solution (buffer) alone. Thus, wherever the titer in salt solution alone is high, even small percentages of serum greatly diminish that titer. Wherever the titer in salt solution is low (less than N/320), small percentages of added serum leave the titer practically unchanged.

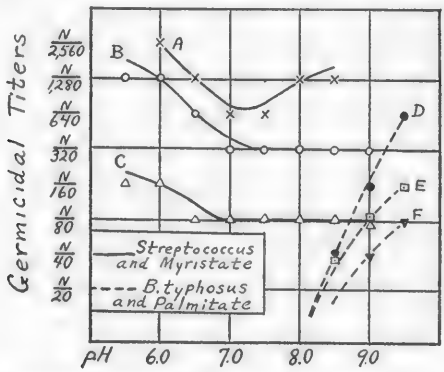

FIG. 2. The germicidal titers of myristate for *Streptococcus: A*, salt control; *B*, 10 per cent serum; *C*, 60 per cent serum. The germicidal titers of palmitate for *B. typhosus: D*, salt control; *E*, 10 per cent serum; *F*, 60 per cent serum. The incubation period was 2 hours at 37°C.

Thus small additions of serum flatten the curves and make them more nearly horizontal. If further larger amounts of serum are added, the titers are reduced all along the line.

In the combination of *Staphylococcus* with laurate, shown in Fig. 1, the salt solution control makes a steep curve; the ratio of the titer at pH 5.0 to the titer at pH 9.0 is 256:1. The addition of 5 per cent of serum to the soap diminishes the titer at pH 5.0 from N/20,480 to N/1,280, while the titer at pH 8.0 to 9.0 is unchanged, and the ratio is now only 16:1. Further additions of serum now diminish the titer equally at all pH; the curves for 5, 10, 20, and 60 per cent

of serum are parallel. Other combinations that, in the presence of serum, gave curves similar to those of Fig. 1 were: *Staphylococcus* with caprylate, caprate, and myristate; *B. typhosus* with caprylate and caprate; *B. diphtheriæ* with caprylate, caprate, and laurate; *Streptococcus* with caprylate, caprate, and laurate.

In the combination of *Streptococcus* with myristate, shown in Fig. 2, the control curve for salt solution is comparatively horizontal.

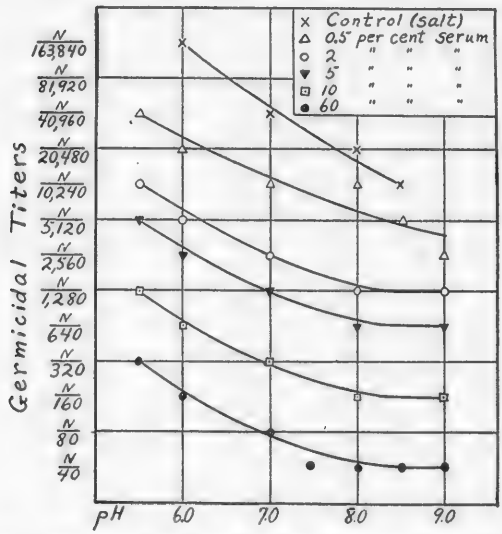

FIG. 3. The germicidal titers of oleate for *Streptococcus*. The incubation period was 2 hours at 37°C.

The addition of serum reduces the titer approximately equally at every pH, so that the serum-soap curves have about the same shape and inclination as the control. The following combinations behaved similarly to this one in the presence of serum: *Streptococcus* with palmitate, and *B. diphtheriæ* with myristate and palmitate.

In Fig. 2 are also shown the curves for *B. typhosus* and palmitate. Here the curve of the control rises steeply toward the alkaline side; the addition of serum inclines the curve toward the horizontal. Similar results were obtained with *B. typhosus* and laurate, myristate, and oleate.

Fig. 3 gives the effect of serum upon *Streptococcus* and oleate. Here the titer in salt solution is high at every pH, though highest in the acid range. Even 0.5 per cent of serum diminishes the titer from N/163,840 to N/10,240 at pH 6.0, and from N/10,240 to N/5,120 at pH 8.0 and 8.5. Further additions of serum diminish the titer equally at every pH.

It is an interesting fact that serum increases both the acid and the alkali tolerance of bacteria. Thus, in salt solution (phosphate-glycine buffer) this strain of *Streptococcus* invariably dies within 30 minutes at pH 5.5 and pH 9.0 at 37°C., while in 0.5 per cent serum it survives for 18 hours at the same reactions. (This organism has become more sensitive to acid and alkali during the past year, hence the tolerance is now different from that reported previously.)

DISCUSSION.

Several investigators have attempted to explain the mechanism of the inhibiting effect of serum upon soap. Von Liebermann (1907) ascribed the inhibitory action to the calcium of the serum, and von Liebermann and von Fenyvessy (1909) suggested both calcium and cholesterol as active constituents of the serum. Sachs (1908), on the other hand, believed the serum proteins to be responsible. Von Korányi (1908) showed that the addition of serum to soap solution caused a slow increase of the surface tension of the soap solution, and suggested this as the cause of the inhibitory effect of the serum. Walker (1924) again suggested calcium as the effective substance, although Lamar (1911) had denied this, and Clark, Zinck, and Evans (1921) stated that calcium played only a minor part in the inhibitory action of serum.

Inasmuch as the mechanism of soap inhibition by serum is of considerable interest, this problem was taken up. Preliminary experiments showed that the active principle was heat-stable; when diluted and boiled, serum is still inhibitory. The active principle does not diffuse through collodion membranes whose permeability is such that they hold protein back. When serum is fractionated by dialysis or by ammonium sulfate precipitation, each fraction is active, and the activity varies approximately with the protein content of that fraction.

The calcium of the serum undoubtedly plays some part, but a minor one, as was also found by Clark, Zinck, and Evans (1921) in their studies on soap hemolysis. When oxalated serum was compared with normal serum, the germicidal titers with the two sera were exactly the same at every pH in 6 experiments, as follows: *Streptococcus* with laurate and myristate; *Staphylococcus* with laurate and myristate; *B. typhosus* with laurate and oleate. In 3 other experiments, the oxalated serum gave a soap titer twice as high as the normal serum at every pH, as follows: *Streptococcus* with oleate (2 experiments) and *Streptococcus* with laurate. A 10 per cent concentration of serum was used for these experiments.

TABLE I.

Germicidal Titers of Potassium Laurate for Staphylococcus in the Presence of Calcium Chloride.

pH	Acetate-asparagine buffer			Phosphate-glycine buffer		
	Control	1–5,000 $CaCl_2$	1–1,250 $CaCl_2$	Control	1–10,000 $CaCl_2$	1–2,000 $CaCl_2$
5.0	N/20,480	N/20,480	N/20,480	N/20,480	N/20,480	N/20,480
6.0	N/5,120	N/5,120	N/320	N/5,120	N/5,120	N/160
7.0	N/1,280	N/320	N/80	N/1,280	N/320	N/40
8.0	N/320	N/160	N/20	N/320	N/160	N/40
9.0	N/160	N/80	N/20	N/160	N/80	N/40

The period of incubation was 2 hours; the temperature, 37°C.

In another series of experiments, varying quantities of $CaCl_2$ were added to buffer solutions without serum, and the soap titers determined. As the phosphate-glycine buffer precipitates the calcium in neutral and alkaline reactions, parallel experiments were undertaken with a special buffer mixture containing N/10 potassium acetate and N/10 asparagine. This buffer was adjusted to the desired pH by means of acetic acid or potassium hydroxide. As is shown in Table I, concentrations of 1–5,000 to 1–10,000 of $CaCl_2$ definitely lower the titer, but only in the neutral and alkaline ranges. In this respect the effect is quite different from that of serum. Larger amounts of calcium produce an even more decided lowering of the titer, except at pH 5.0.

Table I shows that it does not make any difference whether the buffer precipitates the calcium or not. In the case of the phosphate-glycine buffer, either a double decomposition occurs, and calcium laurate is formed, or the precipitated calcium phosphate adsorbs the laurate; in either case the soap is removed from solution.

The amount of calcium in human blood serum is given as 10 to 12 mg. per 100 cc. If it is assumed that sheep serum contains the same amount, then a 60 per cent serum would contain as much calcium as a 1–6,000 solution of $CaCl_2$; while 10 per cent serum should contain as much as a 1–36,000 solution. From Table I it is apparent that such small amounts of calcium can play only a very minor part in the serum inhibition of soaps, and then only in the neutral and alkaline reactions.

The serum protein is undoubtedly more important. Unfortunately this cannot be conclusively proved directly, as no method is known for completely freeing serum protein from lipoid without at the same time denaturating the protein and making it water-insoluble. Extraction with ether, as is well known (Maclean, 1918), removes only a small part of the lipoid. A method which removes a large part of the serum lipoid without denaturating the protein is that of Hardy and Gardiner (1910). This method, modified somewhat to serve the present purpose, is as follows:

To 1 volume of serum at 0°C., 12 volumes of absolute alcohol at 0°C. are added. The mixture is held at 0°C. for 1 hour, with occasional shaking; it is then filtered with suction. The precipitate is washed, first with a mixture of cold alcohol and ether, then with pure ether. In all these operations, the temperature is kept at or near 0°C. A dry powder is obtained which is readily and completely soluble in N/10 NaCl solution.

In Figs. 4 and 5 are shown the titers given by such a "defatted" serum having a concentration of 0.7 per cent dry protein (representing 10 per cent of original serum). The acid titers are somewhat higher in both cases than in 10 per cent normal serum, while the alkaline titers are only a little higher. It should be remembered that this method does not remove all the serum lipoid; repeated extraction with hot alcohol is necessary for complete defatting. Hence it is impossible to tell whether the activity of this partially defatted serum

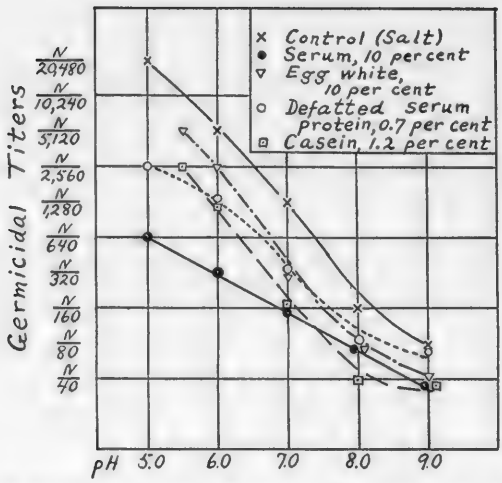

FIG. 4. The germicidal titers of laurate for *Staphylococcus* in the presence of various proteins. The incubation period was 2 hours at 37°C. Crystalline egg albumin in a concentration of 1.5 per cent; gelatin, 2 per cent; and peptone, 2 per cent, each gave a curve exactly like that of the salt control.

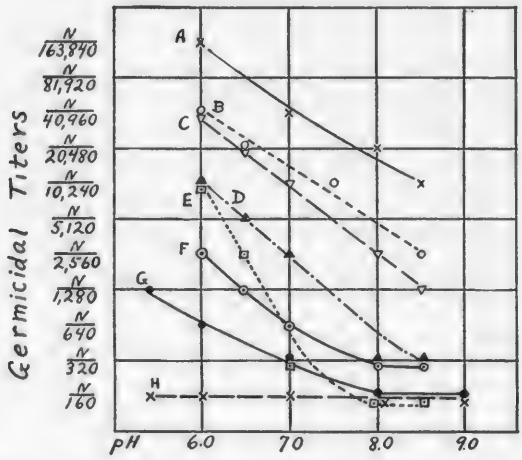

FIG. 5. The germicidal titers of oleate for *Streptococcus* in the presence of various proteins. *A*, salt control; *B*, crystalline egg albumin, 1.5 per cent; also peptone, 2 per cent; *C*, gelatin, 2 per cent; *D*, egg white, 10 per cent; *E*, casein, 1.2 per cent; *F*, defatted serum protein, 0.7 per cent; *G*, serum, 10 per cent; *H*, egg yolk, 1 per cent. The period of incubation was 2 hours at 37°C.

represents the true activity of the protein or that of the residual lipoids.

A number of other protein substances were tested (Figs. 4 and 5). Unaltered egg white, in a concentration of 10 per cent, gave quite different curves from 10 per cent serum, the titers in acid reactions being very much higher than with serum. Crystalline egg white was prepared by the method of Hopkins and Pinkus (1898), recrystallized three times, and dialyzed. In a concentration of 1.5 per cent, it showed no inhibitory action whatever for laurate with *Staphylococcus*, and only slight inhibition of oleate with *Streptococcus* (Fig. 5). Clark, Zinck, and Evans (1921), using crystalline egg albumin in hemolytic experiments with sodium oleate, found that this substance, instead of inhibiting, augmented hemolysis by the soap; this was probably a pH effect, as the crystalline albumin is acid.

Gelatin (freed from calcium by the method of Loeb (1919)), in 2 per cent concentration, and peptone (Parke, Davis and Co.), also in 2 per cent concentration, behaved like the crystallized egg albumin; no effect of these substances was noticed on the action of laurate with *Staphylococcus*, while they only slightly inhibited oleate with *Streptococcus* (Fig. 5).

Casein was prepared from fresh milk by the method of Van Slyke and Baker (1918), and was carefully freed from lipoid by repeated extraction with warm alcohol and ether. In a concentration of 0.75 to 2 per cent, it proved to be an actively inhibitory substance, but the curves are decidedly different from those of serum (Figs. 4 and 5). The alcohol-soluble protein associated with casein (see Osborne and Wakeman, 1918), when suspended in the buffer solutions, proved to be very strongly inhibitory to the germicidal action of soaps.

Egg yolk also was found to be a powerfully inhibitory substance (Fig. 5). With only 1 per cent egg yolk, the titer of laurate for *Staphylococcus* was reduced to N/80 at every pH between 5.0 and 9.0 (not shown in the figure). The activity of egg yolk is probably mostly due to its high lipoid content.

Washed red cells, added to the soap solutions, gave curves almost identical with those of serum.

From these experiments it will be seen that every protein substance tested was more or less inhibitory to the oleate-*Streptococcus* combina-

tion; crystalline egg albumin, gelatin, and peptone, however, failed to inhibit the action of laurate upon *Staphylococcus*. It is undoubtedly a significant fact that all the curves obtained for protein substances run more nearly parallel to the curve for salt solution than they do to the curves for serum.

The lipoids of the serum have hitherto not been definitely related to the soap-inhibiting property. Von Liebermann and Fenyvessy (1909), it is true, reported that cholesterol diminished the hemolytic power of sodium oleate, but Clark and Evans (1921), with more accurate titrations, failed to confirm this. Sachs (1908) extracted lipoids from serum by means of ether, and found that the lipoids alone had no effect on soap hemolysis. Clark and Evans (1921) found the petroleum ether extractives of serum to be inactive, as likewise commercial lecithin.

The experiments shown in Figs. 6 to 8, however, indicate that the lipoids are actively inhibitory to the action of soaps. In the experiments of Figs. 6 and 7, the lipoids were tested in the form of aqueous emulsions, which were prepared as follows:

1. Cholesterol, cholesteryl oleate, and olive oil were made up in a 0.5 per cent solution in acetone and added to the soap dilutions. The acetone was then driven off by heat, and the lipoid-soap emulsions added to the inoculated buffers. The cholesteryl oleate was prepared by the method of Hürthle (1895).

2. Lecithin was prepared from egg yolks, as commercial lecithin was found to be very impure. The yolks were dried, by two extractions with acetone; then they were extracted twice with alcohol at 40°C. The alcoholic extracts were united and evaporated at 45°C.; the residue was dissolved in ether, filtered, and precipitated with acetone. This precipitate was then twice dissolved in ether and precipitated with acetone. The final product was very pale yellow in color and dissolved completely in alcohol. It probably contained considerable cephalin; but as cephalin is also found in serum, there seemed to be no reason for removing it. This lecithin was readily emulsified by stirring in water.

3. Oleic acid emulsion was prepared by adding HCl to sodium oleate solution. Oleic acid and sodium oleate are practically non-toxic for *Staphylococcus* (Walker, 1924; Eggerth, 1926).

4. The serum lipoids were prepared by extracting serum several times with 5 volumes of warm alcohol and then ether: the filtrates were evaporated and the residue dissolved in alcohol and filtered. The alcoholic solution was evaporated, with additions of water from time to time. When free from alcohol, the volume was made up to that of the original serum.

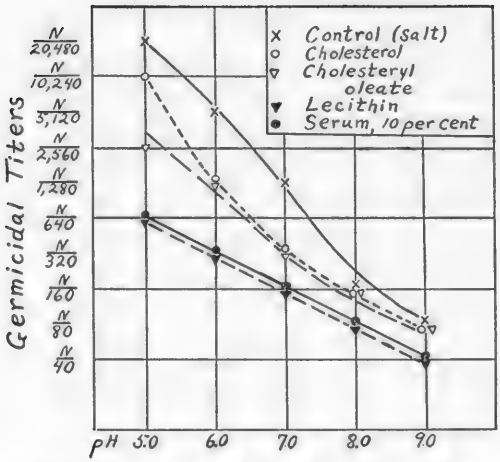

FIG. 6. The germicidal titers of laurate for *Staphylococcus* in the presence of lipoid emulsions. The lipoid emulsions shown are all in a concentration of 1–1,000. An oleic acid emulsion of the same concentration gave a curve identical with that shown for cholesteryl oleate, as did also olive oil. Serum lipoids, in a concentration equivalent to 10 per cent serum, also gave a curve identical with that for cholesteryl oleate. The incubation period was 2 hours at 37°C.

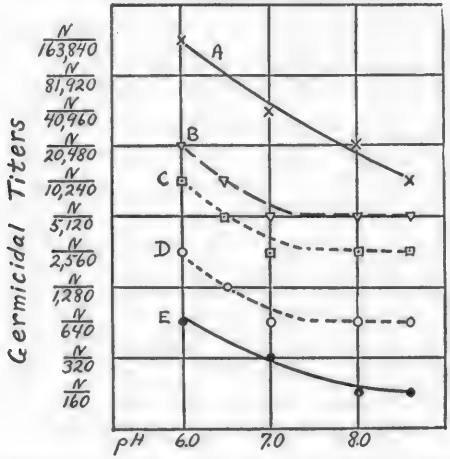

FIG. 7. The germicidal titers of oleate for *Streptococcus* in the presence of lipoid emulsions. *A*, salt control; *B*, cholesteryl oleate, 1–1,000; also olive oil, 1-1,000; *C*, lecithin, 1-5,000; *D*, lecithin, 1-1,000; *E*, serum, 10 per cent. The incubation period was 2 hours at 37°C.

Figs. 6 and 7 show that the lipoids in aqueous emulsions all inhibit the action of soaps. This is especially true of lecithin. A 1–1,000 lecithin emulsion gave a curve identical with that of 10 per cent serum (Fig. 6). Even very dilute emulsions of lecithin were appreciably active; thus, a 1–8,000 emulsion gave a curve identical with that of 1–1,000 cholesteryl oleate (Fig. 6). This outstanding activity of lecithin is undoubtedly due to the basic nature of its molecule, which favors union with the fatty acids.

As the lipoids in serum are present as a highly dispersed colloidal solution, it seemed desirable to test them in aqueous solution. Two such preparations could be obtained. (1) Oleic acid is highly soluble in casein solutions. A 5 per cent casein solution containing N/40 sodium oleate remains perfectly clear when brought to pH 5.5, whereas the solubility of oleic acid in buffer solutions at pH 5.5 is N/5,120. In the experiments shown in Fig. 8, the test fluids contained 0.75 per cent of casein and N/320 of oleate; they remained water-clear at all reactions indicated. (2) Lecithin is highly soluble in bile salts, as shown by Long and Gephart (1908). A sample of sodium taurocholate (Eimer and Amend, "pure") was found to contain an ether-soluble impurity which interfered with the solution of lecithin and was also toxic for *Streptococcus*. The commercial preparation was purified by dissolving it in alcohol containing 2 per cent of sulfuric acid, then precipitating with ether. The precipitate was twice dissolved in alcohol and precipitated with ether, then dried over sulfuric acid. A concentrated solution of taurocholic acid or its salt will dissolve a large quantity of lecithin. Long and Gephart (1908) state that 5 gm. of "bile salt" will dissolve 4.2 gm. of lecithin; with the preparations used in these experiments, even larger amounts of lecithin were readily dissolved. The solutions are perfectly clear and stable at every pH tested and at all dilutions.

The results of these experiments are shown in Fig. 8. The solution of oleic acid-sodium oleate in casein is more inhibitory to the germicidal action of laurate upon *Staphylococcus* than casein alone or the oleic acid-sodium oleate alone in emulsion form (Fig. 6). The taurocholate alone is quite indifferent to the *Staphylococcus*-laurate combination in the concentration tested (1–500); but when lecithin, in a concentration of 1–750, is dissolved in the taurocholate, the

inhibition is pronounced. The lecithin in solution is more active than the lecithin in emulsion (Fig. 6).

The curve for *Streptococcus* and oleate with taurocholate is rather peculiar. This is because the taurocholate, in a concentration of 1–500, is itself germicidal to this *Streptococcus* at pH 6.0. At pH 6.5 it is not germicidal, but its toxicity is added to that of the soap,

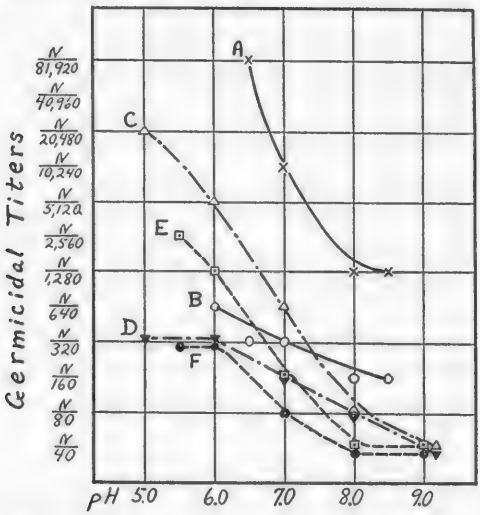

FIG. 8. The germicidal titers of soaps in the presence of lipoids in solution. *A*, titers of oleate for *Streptococcus* in the presence of 1–500 taurocholate (control); *B*, titers of oleate for *Streptococcus* in the presence of lecithin, 1–750, dissolved in 1–500 taurocholate. *C*, titers of laurate for *Staphylococcus* in the presence of 1-500 taurocholate (control); *D*, titers of laurate for *Staphylococcus* in the presence of lecithin, 1–750, dissolved in 1-500 taurocholate. *E*, titers of laurate for *Staphylococcus* in the presence of 0.75 per cent casein (control); *F*, titers of laurate for *Stapylococcus* in the presence of 1–1,000 oleic acid dissolved in 0.75 per cent casein. The incubation period was 2 hours at 37°C.

giving a very high soap titer. At pH 8.0 and 8.5, the taurocholate definitely inhibits the germicidal action of the oleate. But when lecithin was dissolved in the taurocholate solution, a curve was obtained that was identical with that of 10 per cent serum. The lecithin inhibited not only the germicidal action of the oleate, but that of the taurocholate as well.

It was noticed, early in the course of the experiments, that those substances that lower the germicidal titer of the soap, usually increase the solubility of that soap; this increase in solubility was often most marked in the acid reactions. It was often possible to predict the effect of a test substance upon the germicidal titer from the solubility of the soap in that substance. These relationships are shown in Table II.

In determining the solubilities of soaps in serum (Table II) it was found necessary to oxalate the serum to determine solubility at pH 7.0 and more alkaline reactions, otherwise a part of the soap is precipitated by the serum calcium and the results obscured.

A number of interesting facts are brought out by Table II. In the presence of serum at different dilutions and different pH, the ratio of the solubility of the soap to its germicidal titer is remarkably constant, indicating that these phenomena are in some way connected. The corresponding ratios for other test substances vary a great deal; yet it will be observed that every substance that increased the solubility of the soap (or fatty acid) diminished its germicidal titer more or less. The high solubility of oleic acid in casein solution, and of both oleic and lauric acids in lecithin-taurocholate solutions, is noteworthy.

The experiments described above indicate that the serum lipoids and serum proteins both participate in the action of serum upon soap. In unaltered serum it is probable that they largely exist as protein-lipoid conpounds. The question still remains, what is the mechanics of their action upon soaps?

Du Noüy (1922, 1926), in his surface tension studies, interpreted the action of serum upon soaps as being due to an adsorption of soap molecules on the surfaces of the huge serum molecules. In this way the soap is bound so that it can no longer concentrate on other surfaces, and the surface tension of the mixture becomes, in a short time, the same as the surface tension of the serum alone. Even very small quantities of serum, 5 per cent or less, suffice to bring this about.

This explanation might be taken as it stands to account for the effect of serum upon the germicidal action of soaps, were it not for two facts: (1) Under certain conditions, serum inhibits the germicidal action very little or not at all (*e.g.*, the effect of 5 per cent serum on

TABLE II.

The Relation of Solubility to Germicidal Titer.

Test substance	Laurate with *Staphylococcus*				Oleate with *Streptococcus*			
	pH	Solubility	Germicidal titer	Ratio	pH	Solubility	Germicidal titer	Ratio
Phosphate-glycine buffer	5.5	N/5,120	N/10,240	1–2	6.0	N/5,120	N/163,840	1–32
" "	7.0	N/1,280	N/1,280	1–1	7.0	N/5,120	N/40,960	1–8
" "	9.0	N/320	N/80	4–1	8.5	N/640	N/10,240	1–16
Serum, 10 per cent	5.5	N/2,560	N/640	4–1	6.0	N/2,560	N/640	4–1
" 60 " "	5.5	N/640	N/160	4–1	6.0	N/640	N/160	4–1
Oxalated serum, 10 per cent	7.0	N/640	N/160	4–1	7.0	N/1,280	N/640	2–1
" " 60 " "	7.0	N/320	N/40	8–1	7.0	N/640	N/160	4–1
" " 10 " "	9.0	N/160	N/40	4–1	9.0	N/640	N/320	2–1
" " 60 " "	9.0	N/80	N/20	4–1				
Egg white, 10 per cent	5.5	N/5,120	N/5,120	1–1	6.0	N/5,120	N/10,240	1–2
Crystalline egg albumin, 1.5 per cent	5.5	N/5,120	N/10,240	1–2	6.0	N/5,120	N/40,960	1–8
Gelatin, 2 per cent	5.5	N/5,120	N/10,240	1–2	6.0	N/5,120	N/40,960	1–8
Taurocholate, 1–500	5.5	N/5,120	N/10,240	1–2				
Lecithin, 1–750 (in taurocholate)	5.5	N/160	N/320	1–2	6.0	N/320	N/640	1–2
Casein, 1.5 per cent	5.5	N/640	N/2,560	1–4	6.0	N/40	N/2,560	1–64

The solubilities and germicidal titers were determined after 2 hours at 37°C. All test substances were made up in the standard phosphate-glycine buffer. The oxalated serum was prepared by adding 0.5 cc. of 2 per cent ammonium oxalate to 10 cc. of serum and centrifuging until clear.

laurate with *Staphylococcus* at pH 8.0 and 9.0, Fig. 1); and (2) certain substances found by du Noüy to be as active as serum in raising the surface tension of soap solutions, such as gelatin and crystalline egg albumin, have little or no effect upon the germicidal action of soap (Figs. 4 and 5).

In the case where lipoid emulsions are added to soaps (Figs. 6 and 7), it is obvious that the soap will be distributed between three phases: the suspended lipoid, the water, and the bacteria. The concentration of soap in each of these phases will vary, in this particular case, with the solubility of the soap in the respective phases. As the solubility of fatty acids in lipoids is high, then, in acid reactions, the greater proportion of the soap (fatty acid) will be in the suspended lipoid: the concentration of germicide in the bacterial protoplasm will consequently be low, and germicidal action will be greatly diminished. On the other hand, salts of the fatty acids (soaps) are not very soluble in lipoids; hence in alkaline reactions the lipoid phase will contain only a small part of the soap, and the concentration of germicide in the bacteria will be as great or nearly as great as in the salt control; hence germicidal action will not be appreciably inhibited. Figs. 6 and 7 show that this actually occurs. When lipoids in aqueous solution are the test substances (Fig. 8) the results are the same, and it seems likely that the mechanism also is the same.

When serum and bacteria are added to soap solutions the same thing probably occurs. The soap divides itself between three phases: bacterial protoplasm, water, and serum molecules: the final concentration in each phase will vary with the combination attraction of that phase for the soap. Whether the combination with the serum molecules is one of solution of the soap in colloidal micellæ (as with lipoid emulsions) or of chemical combination, or of adsorption in the sense of du Noüy, is immaterial. Crystalline egg albumin and gelatin are less effective than serum because their attraction for soap molecules is less; this is also shown by the fact that neither of them increases the solubility of soap in water.

SUMMARY AND CONCLUSIONS.

1. Far more information about the effect of serum or other substances upon the germicidal action of soaps can be obtained by deter-

mining the germicidal titers over a wide range of pH than by determining the titer at a single pH. In this way a characteristic curve for each test substance is obtained.

2. The curve for a particular concentration of serum bears a definite relationship to the curve for salt solution (buffer) alone. Wherever the titer in salt solution is high, very small amounts of serum greatly diminish that titer. Wherever the titer in salt solution is low, small amounts of serum leave the titer unchanged. Thus small additions of serum flatten the curves and make them more nearly horizontal. If further large amounts of serum are added, a further reduction in titer takes place at all reactions.

3. The calcium of serum has only a very slight effect upon the soap titer.

4. The protein of serum is probably inhibitory to soaps; but the curve for partially defatted serum, and the curves for other protein substances tested, do not run parallel to the serum-soap curves.

5. The various lipoids that are known to be present in serum are inhibitory to the action of soaps, both as emulsions and as clear solutions.

6. The action of serum upon soaps may be regarded as a complex reaction, in which lipoids, protein, and, to a lesser extent, calcium salts take part. Their effect is due to the fact that these substances, by combining with the soaps, remove them from the field of germicidal action.

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